CLINICAL AND GENE EXPRESSION STUDIES OF PALATE DEVELOPMENT

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DECLARATION

I declare that this thesis is my own composition and that the work contained herein was carried out by myself alone unless otherwise stated.

Date 6th June 1991

N.B. Part of the work contained in this thesis has been published elsewhere. Reproductions of these publications are bound in the back of the volume.

To my parents

ABSTRACT

Cleft lip, cleft palate or both are among the most common human congenital malformations with a reported birth prevalence of around 1.5 per 1000 total births in Caucasian populations. In a minority of cases chromosomal aberrations, single gene defects or environmental causes can be identified, but for the majority multifactorial inheritance is suspected although the number and nature of these genes is unknown. Identification of the genes involved in craniofacial morphogenesis can be achieved either directly by detection of stage- and site-specific expression of candidate gene mRNA in the embryo or indirectly by patient linkage or association studies.

In the first part of the study complete ascertainment of facial clefts born in the West of Scotland between 1st January 1980 and 31st December 1984 was attempted. In this five year period 286 cases were identified giving a birth prevalence of 1.53 per 1000 total births. Of these 17.8% had cleft lip only, 51.4% had cleft palate only and 30.8% had both.

Of the 139 patients with cleft lip with or without cleft palate (CL(P)), the lip defect was unilateral in 86/139 (61.9%), midline in 2/139 (1.4%) and bilateral in 41/139 (29.5%), in the remaining 10 cases details of the lip defect were not recorded. There was a statisically significant excess of left-sided cleft lip (55/86) among the unilateral defects (x^2=6.7 $p<0.05$). There was a statistically significant excess of males in the CL(P) group, with a male:female ratio of 1.84:1 (YM x^2=8.36 $p<0.01$). 48/139 (34.5%) of the CL(P) group had congenital abnormalities associated with the facial cleft, 5 of these 48 had a chromosomal aberration, 8/48 a single gene defect, and 8/139 a recognisable malformation syndrome. In the remaining 27 cases no specific aetiology was recognised.

In the cleft palate only group (CP) the male:female ratio was 0.81:1, although the female excess was not statistically significant. 86 of the 147

(57.7%) cases in this group had congenital anomalies associated with their facial cleft. 8/86 had chromosomal anomalies, 12/86 had single gene defects, 30/86 had recognised malformation defects and in 1/86 teratogen induced anomalies. In the remaining 35 cases no specific aetiology was recognised.

Data from the present study are in broad agreement with previous studies from Northern European populations. This study provides an unbiased cohort of patients that can be used in future haplotype association studies and to calculate empiric recurrence risks for use in counselling parents of affected children. It also confirms the remarkable degree of clinical heterogeneity within orofacial clefts group.

In the second part of the study was an attempt to identify the function of a group of 'candidate' genes in normal palatogenesis. To this end the *in situ* hybridisation technique was employed to study the role of transforming growth factors type beta (TGFß) isoforms in murine palatogenesis. Embryos from NIH/Parkes crossed mice were obtained at the critical stages in development of the secondary palate (gestation day (GD) 11.5, 12.5, 13.5, 14.0, 14.5 and 15.0) and hybridised with S^{35}-labelled antisense riboprobes to TGFß1, ß2 and ß3 gene transcripts. A differential expression pattern for all three of these growth factors was established during palate development.

No specific pattern of hybridisation was found until GD 13.5 when TGFß3 RNA was detected in the medial edge epithelia (MEE) of the vertical palatal shelves and in the epithelia of the nasal septum that will go on to fuse with the shelves. After elevation of the shelves TGFß3 expression continued to be specific to the MEE and could be detected in the cells of the midline epithelial seam immeditely after fusion. The TGFß3 hybridisation signal in MEE at this stage is the most easily visible of any in the whole embryo, it is however almost completely absent from the medial edge palatal mesenchyme. As the epithelial seam disrupts by epithelial-mesenchymal transformation the specific pattern of TGFß3 expression is lost. TGFß1 expression followed a similar pattern of

expression to TGFß3 but was not detectable in the MEE until GD 14.0, with an apparently much less abundant transcript. Specific hybridisation of TGFß1 was also lost with disruption of the epithelial seam. TGFß2 expression followed a different pattern first detectable in discrete areas of hyperplasia in the oral epithelium of the vertical palatal shelves, these probably represent the developing palatal rugae. With elevation of the shelves TGFß2 hybridises specifically to the medial edge palatal mesenchyme. As fusion occurs expression is also seen in the mesenchyme underlying the nasal epithelium. No hybridisation of TGFß2 is seen in the midline epithelial seam.

This pattern of site- and stage- specific hybridisation inferred an important role for these isoforms in the normal processes of palatal fusion. To test this hypothesis the experiments were repeated in retinoic acid treated embryos that would develop cleft palate. Pregnant C57/B mice were treated with either 100mg/Kg or 200mg/Kg of all-trans retinoic acid at 12.0gd or 14.0gd. Although the palatal shelves of all the treated embryos were obviously hypoplastic and had delayed shelf elevation there was no alteration in the expression patterns of TGFß1, ß2 or ß3.

Knowledge of the *in vitro* properties of the TGFßs and the RNA localisation data argue an important role for TGFß1, ß2 and ß3 in mammalian palatogenesis. It is however interesting that failure of fusion can occur with apparently normal expression patterns. It seems likely that these growth factors act via an alteration in the extracellular matrix of the medial edge palatal mesenchyme thus providing a suitable microenvironment for epithelial transformation and mesenchymal migration. Although further work will be necessary to elucidate the role of the TGFßs in palatogenesis this will be greatly helped by transgenic techniques that offer the possibility of stage and site specific modulation of gene action during morphogenetic processes. It must be hoped that by understanding the molecular mechanisms involved in all developmental processes that strategies for preventing congenital malformations can be developed.

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ABBREVIATIONS

aa	amino acid
ATP	adenosine triphosphate
bp	base pair
BMP	bone morphogenetic protein
cDNA	complementary DNA (made from mRNA)
cRNA	complementary RNA (transcribed from DNA in a transcription vector; also referred to as "riboprobe")
CTP	cytidine triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FGF	fibroblast growth factor
g	gram
GAG	glycosaminoglycans
GTP	guanine triphosphate
IGF	Insulin-like growth factor
HBGF	Heparin-binding growth factor
kb	Kilobase
MEE	medial edge epithelium
MEPM	medial edge palatal mesenchyme
mRNA	messenger RNA
NC	neural crest
PAGE	polyacrylamide gel electrophoresis
p.c.	post coitum
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor

mg	milligram
ml	millilitre
mM	millimolar
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar
ng	nanogram
nM	nanomolar
OD	optical density
pg	picogram
pM	picomolar
rpm	revolutions per minute
rRNA	ribosomal RNA
RNA	ribonucleic acid
SDS	sodiumdodecylsulphate
TEMED	N,N,N',N'-Tetramethylethylenediamine
TESPA	3-aminopropyltriethoxysilane
TGF	transforming growth factor
Tris	Tris (hydroxymethyl) aminomethane
tRNA	transfer RNA
TTP	thymidine triphosphate
UTP	uridine triphosphate
v/v	volume per volume
w/v	weight per volume
YM x^2	Yates modification of the chi-squared test

CHAPTER 1 INTRODUCTION

1.1 FACIAL CLEFTS

Cleft lip, cleft palate or both are among the most common congenital malformations in man (de Wals & Lechat 1986) and although they are generally considered treatable conditions, therapy often involves complex surgical procedures carried out in specialist centres with the necessity of long term follow-up by a multidisciplinary team. It would seem reasonable, therefore, to describe these malformations as major congenital anomalies (Czeizel & Tusnady 1984).

Most facial clefts are either cleft lip with or without cleft palate (CL(P)) or isolated cleft palate (CP). In addition, however, there is a smaller group of unusual facial clefts such as oblique and lateral facial clefts. These are much rarer and, almost certainly, aetiologically distinct, this subgroup will therefore not be discussed further (for review see Gorlin *et al.* 1990).

The two major subgroups of facial cleft malformations, CL(P) and CP, have generally been considered as separate genetic and developmental entities (Fogh-Andersen, 1942; Fraser, 1970; Gorlin *et al.* 1990). The basis of this distinction has been from both embryological studies (see section 1.2.3) and family recurrence data. Poul Fogh-Andersen (1942) was the first to recognise these genetic differences in a study of a large number of Danish families with one member or more affected with cleft lip, cleft palate or both. He reported that siblings of patients with CL(P) have a higher incidence of cleft lip (CL) and cleft lip and palate (CL+P) but not of CP and that this homogeneity of defect recurrence also occurred in the siblings of CP patients. Subsequent reports have confirmed these findings (Fujino *et al.* 1963; Woolfe, 1971; Bonaiti *et al.* 1982; Czeizel & Tusnady 1984). Although these data have been generally accepted, it is interesting to note that exceptions exist to this recurrence phenomenon. Families with Van der Woude syndrome, an autosomal dominant condition, can have both types of facial cleft defect associated with lip pits, segregating with the gene (Woolf, 1971). Woolf (1971) has also shown a small but convincing increase in the

incidence of CP occurring in the families of a large cohort of patients with CL(P).

1.1.1 The epidemiology of facial clefts

Facial clefts are predominantly non-lethal malformations that are usually obvious at birth or in early neonatal life with surgical treatment as the only therapeutic option. These anomalies would, therefore, seem to present investigators with few problems in the estimation of accurate birth prevalence figures. This has prompted many CL(P) and CP birth prevalence studies which have used either birth certificates (Woolf & Woolf, 1963; Tenconi *et al.* 1988) or hospital records (Fogh-Andersen 1942; Bear 1976; Bonaiti *et al.* 1982) as their main sources of ascertaining the affected individuals. This single-source approach to ascertainment has, however, been criticised on two accounts. Firstly, it has been estimated that in up to one third of cases the presence of the facial cleft will not be recorded on birth records or neonatal discharge forms (Shapiro, 1976). Secondly, Drillien *et al.* (1966) have shown that up to 20% of infants with facial clefts will die before operation or before the cleft is recognised. It must be assumed, therefore, that using either of the above methods alone would lead to significant under-ascertainment of facial cleft cases.

From published reports the average birth prevalence of facial clefting in Caucasian populations is 1 per 1000 total births for CL(P) and 1 per 2000 total births for CP (for reviews see; Shapiro, 1976; Gorlin *et al.* 1990). Cleft lip accounts for 20-30% of the whole group, cleft palate 30-45% and both 35-50% (Drillien *et al.* 1966). As more studies have been published, significant racial differences in birth prevalence for all facial clefts have become apparent, with a higher birth prevalence in peoples of oriental origin: 2.64 per 1000 total births in Japanese (Neel, 1958) and 3.17 per 1000 total births in North American Indians (Tretsven, 1963) and a lower birth prevalence in African Americans, at between 0.59-0.82 per 1000 total births (Longnecker *et al.* 1965; Chung & Myrianthopoulos 1968). The majority of these inter-racial variations in the facial clefts group are, however, accounted for by differences in the CL(P) birth prevalence which range from 0.42 per

1000 total births in African Americans, to 2.7 per 1000 total births in Canadian Indians. Racial differences are not so apparent in the CP group (Chung & Mirianthopoulus, 1968; Neel, 1958) with an incidence of 0.41 per 1000 total births in Black Americans(Chung & Myrianthopoulos, 1968), 0.44 per 1000 total births in Canadian Indians (Lowry & Renwick, 1969) and an average birth prevalence of 0.5 per 1000 total births in European populations (Shapiro, 1976).

Ching & Chung (1974) have shown that the racial differences in CL(P) birth prevalence are likely to have a genetic basis. In an extensive study from Hawaii, they have shown that Japanese immigrants continue to have increased birth prevalence of CL(P) and by studying inter-racial crosses have shown that Caucasian-Japanese matings have intermediate birth prevalence, thus suggesting that the racial differences are independant of environment. Support for this theory is provided by Leck (1972) who has shown that the variation in the birth prevalence of CL(P) between different ethnic groups living in the same areas is eight-times greater than that among geographically scattered populations of the same ethnic origin.

The differences in sex ratios within the facial cleft groups have proven to be more complicated, varying with severity of the cleft, the number of affected sibling in a family and racial origin. In all studies of Caucasian populations CL(P) occurs more frequently in males than females with an average male to female ratio of 2:1 (Shapiro, 1976). In Japanese populations there is a significant male excess in the cleft lip and palate group but not in the cleft lip only group (Fujino *et al.* 1963). In Caucasians the male excess in the CL(P) group becomes more apparent with increasing severity of cleft (Fogh-Andersen, 1942) and less apparent when more than one sibling is affected in the family (Niswander *et al.* 1972). In both races there would appear to be a slight excess of affected females in the CP group (Fraser, 1970; Shapiro, 1976). In African Americans there is no overall significant sex difference in either group (Shapiro, 1976), although isolated cleft lip would appear to be more common in females (Green *et al.* 1964). No generally accepted explanation for these gender differences exists, although sex differences in the timing of critical developmental stages in craniofacial

development are thought to have an as yet undefined role in their aetiology (Burdi & Silvey, 1969)(see section 4.1).

The differences in laterality of the cleft lip group has also proved to be a consistent and puzzling finding. Of unilateral CL (85% of all CL) two thirds have left sided defects regardless of sex, race and severity of defect (Fogh-Anderson, 1942; Fraser & Calnan, 1961). No convincing explanation for these differences has been advanced (see section 4.1).

1.1.2 Family studies

Poul Fogh-Andersons' (1942) classic study of 703 probands with CL, CP or both showed a six-fold increase in the incidence of facial clefting in their third degree relatives or closer. All subsequent studies have confirmed this familial clustering (for review see Fraser, 1970; Shapiro, 1976). Twin studies have shown a significantly higher concordance of clefts in monozygous than dizygous pairs, the heritability (h^2) has been estimated at 0.35 (i.e. 35% of the difference between those with clefts and those without may be attributable to genetic factors) (Hay & Wehrung, 1970).

The analyses of family data in non-mendelian diseases have improved greatly over the last thirty years helped by the improvement in computer hardware capabilities, the work of the so-called English School (Edwards 1960; Carter 1965) and the group working at the University of Hawaii (Morton, 1959; Morton & MacLean, 1974; Lalouel *et al.* 1983). Edwards (1960) and Carter (1965) championed the multifactorial (quasicontinuous) model of inheritance in facial clefting that has been supported by several extensive studies of family data (Woolf, 1971; Czeizel & Tusnady 1972; Dan-ning *et al.* 1982). This model postulates that the cumulative effect of numerous weak, independent factors, genetic or environmental, determines liability to clefting, which occurs when liability exceeds a threshold. More recent analyses and reanalyses of family data have been performed under mixed (Morton & McLean, 1974) or unified (Lalouel *et al.* 1983) models of inheritance, which combine two conceptually different models, the multifactorial and generalised single locus model (Demenais *et al.* 1984;

Chung *et al.* 1986). These analyses have failed to discriminate between polygenic and monogenic inheritance within the whole group. Chung *et al.* (1986), however, suggested that both modes of inheritance were operating in CL(P) after studying 2,998 nuclear families from Denmark, with a single recessive locus accounting for about one third of cases. Interestingly the major locus effect was not seen in the Japanese families studied.

1.1.3 Aetiology of Facial clefts

Both CL(P) and CP are anomalies with remarkably heterogeneous aetiologies. Within both of these groups there are well recognised unifactorial aetiologies (chromosomal, single gene and teratogenic), the majority of cases have, however, no specific cause that can be identified (Gorlin *et al.* 1990). Due to the complex nature of the mechanical and biochemical events involved in palatogenesis (see section 1.3), it would seem likely that the processes of palate development could be interrupted at many different stages by a wide variety of factors with cleft lip, cleft palate or both as a consequence. The number and nature of these disruptive factors is not known, though our understanding of the normal developmental processes and the causation of facial clefts may be greatly advanced by the study of the inborn errors of morphogenesis (Opitz *et al.* 1979).

1.1.3.1 Environmental factors

Many specific environmental factors have been implicated in facial clefting (for review see Gorlin *et al.* 1990). Some of these, such as hyperemesis (Drillien *et al.* 1966) and pre-eclampsia (Fraser, 1970) have, however, not been confirmed by subsequent studies (Saxen 1974). Shprintzen *et al.*'s (1985) attempted to quantify the load of environmental disease in the facial cleft population by studying 1000 cases of orofacial clefts. They found 4% of these were due to specific teratogenic agents, of which fetal alcohol syndrome was the most common (75%) entity. The known teratogenic syndromes associated with orofacial clefts in humans are shown in Table I.

In contrast to the relative paucity of information of teratogenesis as a cause

of facial clefts in human subjects, attempts at chemical manipulation of palate development in animal models have provided large amounts of *in vivo* teratogenic data (Shah, 1984). Retinoic acid (RA) is one of the most widely studied of these agents and is often used as a tool in the study of normal and abnormal palate development. As RA was used as a teratogen in the present study, its *in vivo* effects will be reviewed in detail.

TABLE I Facial cleft syndromes with environmental causes

Syndrome	*Features*
Fetal alcohol	Mental retardation, poor growth and cleft palate
Fetal diazepam	Cleft palate only
Fetal hydantoin	Unusual facies, digital anomalies, congenital heart defects (CHD) and cleft palate
Fetal meprobamate	CHD, CP and limb defects
Fetal methotrexate	Unusual facies, mesomelia and other limb defects
Fetal trimethadione	Mental retardation, CHD, unusual facies and CP
Fetal vitamin A	Ear abnormalities and CNS anomalies (see section 1.1.3.2)
Maternal hyperthermia	Neural tube defects and craniofacial abnormalities
Maternal phenylketonuria	Microcephaly, mental retardation and cleft palate.

1.1.3.2 Retinoic acid

The retinoid group of molecules consist of retinol (Vitamin A) which is an alcohol form that is metabolised to retinal (aldehyde form) and retinoic acid (acid form). The *in vivo* effects of these substances are thought to be mediated

through three groups of molecules, the cellular retinoic acid-binding protein (CRABP), the cellular retinol-binding protein (CRBP)(Ong *et al.* 1982) and the nuclear retinoic acid receptors (RARs)(Zelent *et al.* 1989). Although the retinoids are considered teratogenic at supraphysiological levels, their *in vitro* effects (Glick *et al.* 1989) and the *in vivo* localisation patterns of RA (Thaller & Eichele, 1987), the binding proteins (Perez-Castro *et al.* 1989) and the RARs (Zelent *et al.* 1989) argue an important role for these molecules as morphogens. Retinoic acid in particular, has proven a very useful tool in the study of orofacial development. Cohlan (1953) first reported a specific pattern of craniofacial and limb anomalies in rats exposed to Vitamin A in embryonic life. Subsequently many other studies have catalogued these anomalies in the chick (Tamarin *et al.* 1984), the mouse (Newall & Edwards 1981), simian species (Fantel *et al.* 1977) and humans (Rosa *et al.* 1986). Following marketing of the 13-cis form of retinoic acid for the treatment of severe acne, Rosa *et al.* (1986) reported 44 outcomes of pregnancies from women taking this drug during the first trimester. The commonest defects in this group were, malformations of the central nervous system (37/44), ear abnormalities (30/44), congenital heart defects (22/44), micrognathia (13/44), cleft palate (5/44) and microphthalmia (5/44). These abnormalities are similar to those seen in animal studies.

The pathogenesis of retinoic acid-induced cleft palate has been the subject of much study. Newall and Edwards (1981) have shown that it is possible to induce cleft palate in C57B1 mice by giving large doses only twelve hours prior to fusion. Abbott *et al.* (1989) have suggested that the aetiology of the retinoid-induced cleft varies with the embryonic stage. Those mice treated at gestation day (GD) 10 developed small palatal shelves that did not make midline contact (see section 1.3). The medial edge epithelia (MEE) of these hypoplastic palatal shelves failed to undergo peridermal cell death and differentiated into oral type palatal epithelium. Those embryos treated on GD 12 showed no palatal growth inhibition with the palatal shelves making contact above the tongue. The MEE of these shelves, however, developed a nasal epithelial phenotype with subsequent failure of fusion. It is of interest that the distribution of the epidermal growth factor (EGF) receptor is altered in the palates of retinoic acid exposed embryos (Abbott *et al.* 1988) (see

section 1.4.4). Although the mechanism of retinoid action in the palate is still far from clear, knowledge of their complex actions on mesenchyme proliferation and epithelial differentiation and concommitant changes in growth factor expression pattern may allow informed hypotheses.

1.1.3.3 Chromosomal anomalies causing clefts

Both CL(P) and CP are common in trisomies 13 and 18. In published case reports CL(P) is also frequently associated with abberations involving chromosomes 1q, 3, 4, 7q, 9p, 10p, 11p, 13q and 22q (Schinzel, 1984). CP is also common in many different chromosomal aberrations with those involving 1q, 3, 4, 5q, 6q, 7q, 8q, 10, 13, 14, 17, 18, 22 and X being the most common (De Grouchy, 1984; Schinzel, 1984). The present incomplete understanding of the pathogenesis of abnormalities associated with chromosomal aneuploidy has, however, made interpretation or application of these data very difficult.

1.1.3.4 Single gene defects

Appendix A summarises the conditions with facial clefting as a feature that are listed in McKusicks' catalogue 'Mendelian Inheritance in Man' (1990) and the London Dysmorphology Database (Winter & Barraitser, 1988). There are 53 autosomal dominant, 67 autosomal recessive and 13 X-linked conditions listed in the catalogue. Many of these syndromes have only been reported in one family and may represent so-called 'private' recessive syndromes. In spite of the large number of single gene defects described, as a group they are thought to represent a relatively small proportion of the facial cleft population (Gorlin *et al.* 1976). Recently, Shprintzen *et al.* (1985) have challenged this assumption by suggesting that single gene defects account for a significant proportion of the facial clefts. In a comprehensive hospital based survey of facial clefts they found that four autosomal dominant conditions accounted for 15.2% of the whole group, velo-cardio-facial syndrome (4.7%), Van der Woude syndrome (4.5%), Marshall-Stickler syndrome (3.8%) and Treacher Collins syndrome (2.2%). Other surveys have supported these conditions as important causes of facial

clefts (particularly CP) in hospital populations (McDonald *et al.* 1989; Rollnick & Pruznansky *et al.* 1981).

Attempts at molecular characterisation of the genes involved in the four common genetic causes of facial clefting have, as yet, been unsuccessful. Velo-cardio-facial (VCF) syndrome is an autosomal dominant condition recognised by the characteristic facies, ventricular septal defects and overt or submucous cleft palate (Shprintzen *et al.* 1981). There is no chromosomal localisation for the VCF gene. Van der Woude syndrome is characterised by facial clefting and lower lip pits (Cervenka *et al.* 1967). It has been localised to chromosome 1 (1q25-41) by linkage to the renin gene (Murray *et al.* 1988), although no 'candidate' gene in this area has been recognised. Marshall-Stickler syndrome or hereditary ophthalmoarthropathy, is a pleotropic condition, characterised by wide joints, myopia, deafness and cleft palate (Opitz *et al.* 1972). Francomano *et al.* (1987) demonstrated genetic linkage to the COL2A1 locus (12q13) in two large families with Marshall-Stickler syndrome, however no mutations of this gene have been demonstrated in affected individuals. Treacher Collins syndrome is characterised by lower lid coloboma, hypoplasia of the zygoma, microtia and macrostomia (Fazen *et al.* 1967), it has been the subject of much recent interest as it has striking similarities to retinoic acid embryopathy (Sulik *et al.* 1987) and the malformations in transgenic mice with ectopic expression of the Hox 1.1 gene (Balling *et al.* 1989). Balestrazzi *et al.* (1983) described a girl with Treacher Collins syndrome with a de novo apparently balanced translocation t(5;13); there are no genetic linkage data as yet.

1.1.3.5 Malformation syndromes

Appendix B summarises the currently recognised malformation syndromes of unknown aetiology associated with orofacial clefting (Winter & Barraitster 1988). In two recent studies of abnormalies associated with cleft lip, cleft palate or both, unrecognised malformation compounds accounted for almost a quarter of the cases (Shprintzen *et al.* 1985; Rollnick & Pruzansky, 1981). The recognised malformation syndromes accounted for approximately 10% of the total groups. Isolated Pierre Robin sequence

(micrognathia, U-shaped CP and glossoptosis) is the most common of these, identified in around 4% of the whole group. The primary defect in Pierre Robin sequence (PRS) is thought to be mandibular hypoplasia, with glossal obstruction of palate closure (Clarke *et al.* 1988). The holoprosencephaly, facio-auriculo-vertebral and amniotic band sequences accounted for about 2% each.

1.2 CRANIOFACIAL MORPHOGENESIS

Most studies of vertebrate craniofacial development use non-human species with species-specific embryonic size and gestational periods. To allow comparison between vertebrate species, corresponding developmental stages are assessed using the Carnegie System. This system, developed by Streeter in his classic series of articles on human embryonic development (Streeter, 1942; Streeter, 1945; Streeter, 1948), is summarised in Table II.

As the embryological studies in sections 1.2 and 1.3 describe experimental work on several different mammalian species, the animal model used will be indicated in each case. Although extrapolation of these data to the human must remain speculative, the limited data available on human embryos has yielded no major discrepancies in the anatomical (Andersen & Mathiessen, 1967) or histochemical (Abbott & Pratt, 1987) aspects of secondary palate development with that available from non-human mammalian species. Studies of craniofacial development of avian (Wedden *et al.* 1986) and reptilian (Sharpe & Ferguson, 1988) species have provided much fascinating information of great interest to anyone studying developmental systems. The development of the palate in chicks (having naturally cleft palates) and alligators (palates fuse by epithelial migration) would, however, appear to be fundamentally different from that in mammalian species (for review see Ferguson, 1988) and will, therefore, not be discussed further.

1.2.1 The pharyngeal arches

The pharyngeal arches are bars of tissue that arise ventral to the hindbrain in

TABLE II The Carnegie System of Staging Development

Carnegie stage	Size (mm) (human)	Age (days)	Features
1	0.1	1	Fertilisation
2	0.1-0.2	1.5-3.0	From 2-16 cells
3	0.1-0.2	4	Free blastocyst
4	0.1-0.2	5-6	Attaching blastocyst
5	0.1-0.2	7-12	Implanted although previllous
6	0.2	13	Chorionic villi; primitive streak
7	0.4	16	Notochordal process
8	1.0-1.5	18	Primitive pit
9	1.5-2.5	20	Somites first appear
10	2.0-3.5	22	Neural fold begin to fuse; 2 pharyngeal bars
11	2.5-4.5	24	Rostral neuropore closes
12	3-5	26	Caudal neuropore closes; 3-4 pharyngeal bars; upper limb buds appear
13	4-6	28	Four limb buds; lens disc; otic vesicle
14	5-7	32	Lens pit and optic cup
15	7-9	33	Lens vesicle; nasal pit; antitragus beginning; hand plate
16	8-11	37	Nasal pit faces ventrally; foot plates; auricular hillocks begin
17	11-14	41	Head relatively larger; nasofrontal groove; finger rays
18	13-17	44	Tip of nose distinct; eyelid folds
19	16-18	47.5	Trunk elongating and straightening
20	18-22	50.5	Upper limbs longer and bent at elbows
21	22-24	52	Fingers longer; hands approach each other
22	23-28	54	Eyelids and external ear more developed
23	27-31	56.5	Head more rounded; limbs longer and more developed

Adapted from O'Rahilly & Muller (1987)

human embryos at stages 10-12 (Bartelmez & Evans, 1926). Each consists of a mesenchymal core partly derived from migratory cranial neural crest cells, covered externally by surface ectoderm and internally by epithelia of endodermal origin (Sulik & Schoenwolf, 1985). Although 3-4 pharyngeal arches appear in a rostrocaudal sequence in human embryos (O'Rahilly & Muller, 1987) only the first and second arch contribute directly to facial structures (Table III). The first arch, although initially linear, with differential growth forms the shape of an 'c'. The dorsal part of the arch (maxillary process) forms the rostral arm of the 'c' and the ventral part (mandibular process) forms the caudal arm (Fig.1A) (Streeter, 1945).

In early stage 12 the primitive mouth or stomodeum has been formed, bordered superiorly by the frontal process, laterally by the maxillary processes and inferiorly by the mandibular processes (Fig.1A) (Sulik & Schoenwolf 1985). In stage 13 the mandibular processes enlarge, grow medially and merge in the midline to form the lower lip and mandible. Although facial development involves the formation of many structures only the formation of the nose and upper lip will be dealt with in detail here.

The paired maxillary and mandibular processes together with the frontal process will form the facial structures. In addition to these 5 major processes, the maxillary and frontal components contribute another 3 paired mesenchymal growth centres (medial and lateral nasal ridges and the intermaxillary processes) which will form the structures of the central face (Fig.1A-D). These growth centres are areas of condensed mesenchyme with detatched foci of growth separated from each other by a common underlying matrix of loose connective tissue (O'Rahilly & Muller, 1987). Merging of these prominences involves no ectodermal adsorption but rather 'filling-in' of the furrows between them by mesenchymal proliferation (Peter 1913).

1.2.2 Formation of the nose and nasal cavities

Warbrick (1960) recognised four stages in the formation of the nasal cavities in human embryos. (1) Formation of the nasal placodes at stage 14 as thickenings of the surface ectoderm on either side of the frontal process. (2)

FIGURE 1

DEVELOPMENT OF THE CENTRAL FACE

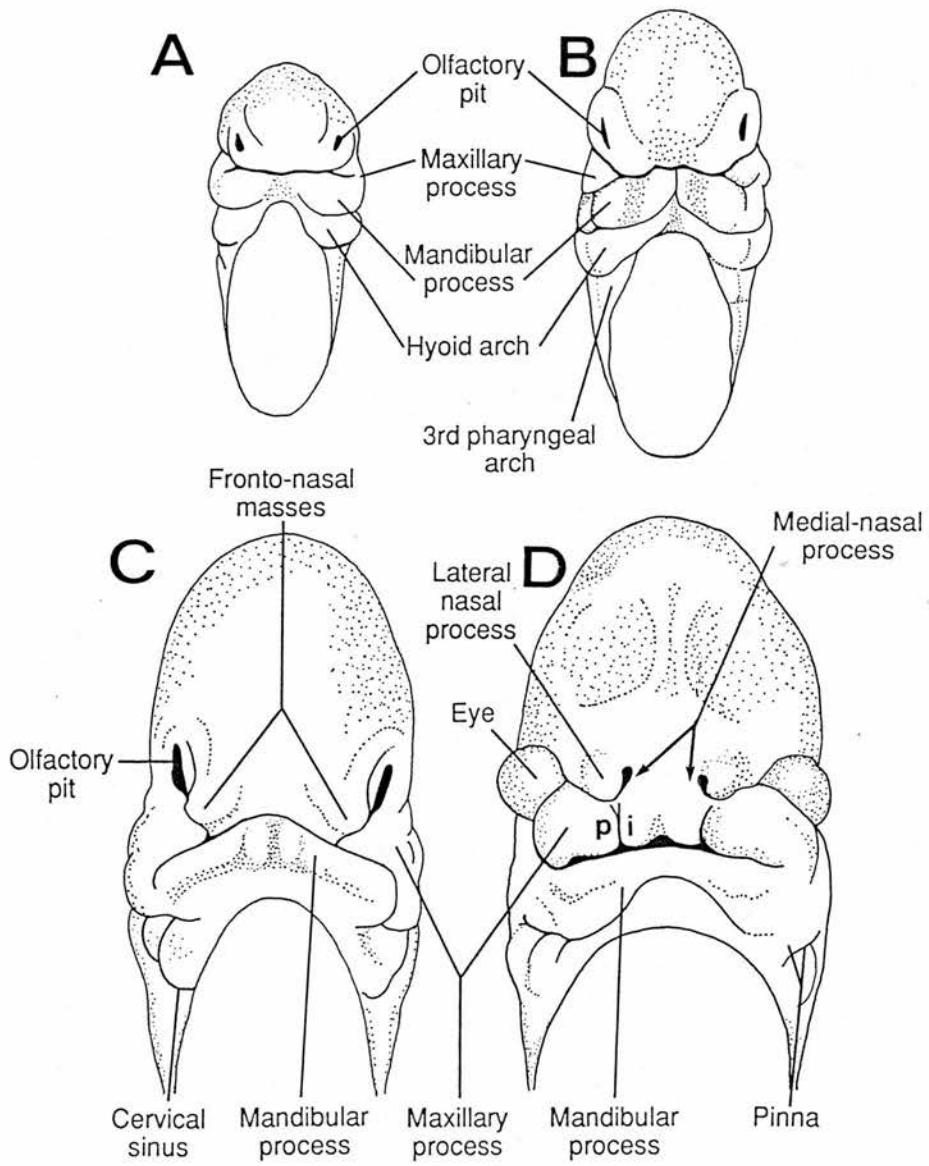


FIGURE 1. Develpment of the central face. Diagrammatic representation of central face development from the frontal process and the paired maxillary and mandibular processes. (A) Represents a human embryo at early Stage 12 with the primitive mouth bordered rostrally by the frontal process, laterally by the maxillary processes and caudally by the mandibular processes. (B) Late in Stage 12 the mandibular processes enlarge until the growth centres are adjacent in the midline. (C) In Stage 13 the mandibular processes fuse and the frontonasal mass becomes more distinct. (D) In Stage 16-17 the medial nasal ridge and the intermaxillary processes (i) can be distinguished as separate growth centres, the intermaxillary centres will fuse with the premaxillary centres (p) late in Stage 17.

Formation of the nasal grooves. (3) Deepening of these grooves to form the nasal pits. (4) Formation of the nasal sacs as the nasal pits deepen and grow towards each other. The ventral sac epithelium (nasal fin) maintains epithelial continuity between the nasal cavity and the roof of the mouth, separating the maxillary and intermaxillary growth centres (Streeter, 1948). The nasal sacs are separated from the primitive mouth by the oronasal membrane, this ruptures to form the primitive choanae (England, 1983). The paranasal air sinuses develop as diverticulae of the lateral nasal wall and extend into the maxilla, frontal, ethmoid and sphenoid bones (Sadler, 1989).

TABLE III Fate of the 1st and 2nd pharyngeal arch mesenchyme

Pharyngeal arch	*Skeletal*	*Muscles*
1st arch:		
maxillary process	premaxilla, maxilla, zygoma and part of the temporal bone	muscles of mastication, the anterior belly of the digastric, mylohyoid, tensor
mandibular process	incus, malleus and mandible	tympani and tensor palatini muscles
2nd arch	stapes, parts of the temporal bone, hyoid bone (lesser horn only)	stapedius, stylohyoid, posterior belly of the digastric, auricular muscles and muscles of facial expression

Three paired facial processes form from the nasal ridges that surround the nasal pits. The lateral portions of the nasal ridges form the lateral nasal processes, most of the medial portion becomes the medial nasal process and the ventral ends of which form the premaxillary centres. The nose proper is thus formed from seven processes, the frontal process gives rise to the

bridge, the merged medial processes form the nasal septum, the premaxillary centres form the median philtrum, columella and tip and the lateral processes form the alae.

1.2.3 The primary palate and upper lip

With continued growth the premaxillary centres merge in the midline to form the intermaxillary segment (Fig.1D). This segment consists of a labial component, which will form the philtrum of the upper lip, an upper jaw component, which carries the four incisor teeth and a palate component, which forms the triangular primary palate. In the first half of this century many embryologists, most notably Fraser (1931), believed that the upper lip was formed in the human embryo by fusion of the maxillary processes in the midline, more recently Warbrick (1960), using wax plate reconstruction, and Sulik and Schoenwolf (1985), using scanning electron micrography, have convincingly demonstrated that the upper lip is formed by merging of the maxillary processes with the intermaxillary segment at stage 17 (Fig.1D).

More detailed studies of the histological processes involved in the fusion of the upper lip have shown that the intermaxillary segment forms an isthmus of mesenchyme that is isolated from the maxillary processes by the epithelial wall of the nasal fins. This mesenchymal mass then expands through the proliferation of neural crest cells with fusion occurring after autolysis of the nasal fin by coalescence of the intermaxillary and maxillary mesenchyme (Andersen & Mathiessen 1967; Trasler, 1968).

1.3 FORMATION OF THE SECONDARY PALATE

After closure of the primary palate at stage 17 the oral cavity is roofed by the frontal process, walled by two lateral maxillary processes, floored by the merged first arches and occupied by the enlarging tongue and the developing nasal septum. It communicates dorsally with the foregut and ventrally with the amniotic cavity via the mouth and the nares. Development of the secondary palate is phenotypically similar in all mammalian species

studied (for review see Ferguson, 1988) and can be summarised as; initiation of the palate primordia, initially vertical growth of the palatal shelves, subsequent elevation and approximation of the shelves, fusion of the medial edge epithelia to form an epithelial seam followed by disruption of the seam establishing mesenchymal continuity (Fig.2A-D).

1.3.1 The palatal primordia and vertical growth

In stage 18 the palatal shelves arise from the bilateral maxillary processes as distinct ridges of mesenchymal cells associated with craniopharyngeal ectoderm (Greene & Pratt 1976). Shah (1984) carried out an ultrastructural study of shelf initiation in hamster embryos, and showed a change in epithelial phenotype from simple cuboidal before the appearance of palatal

FIGURE 2 Normal mammalian palatogenesis

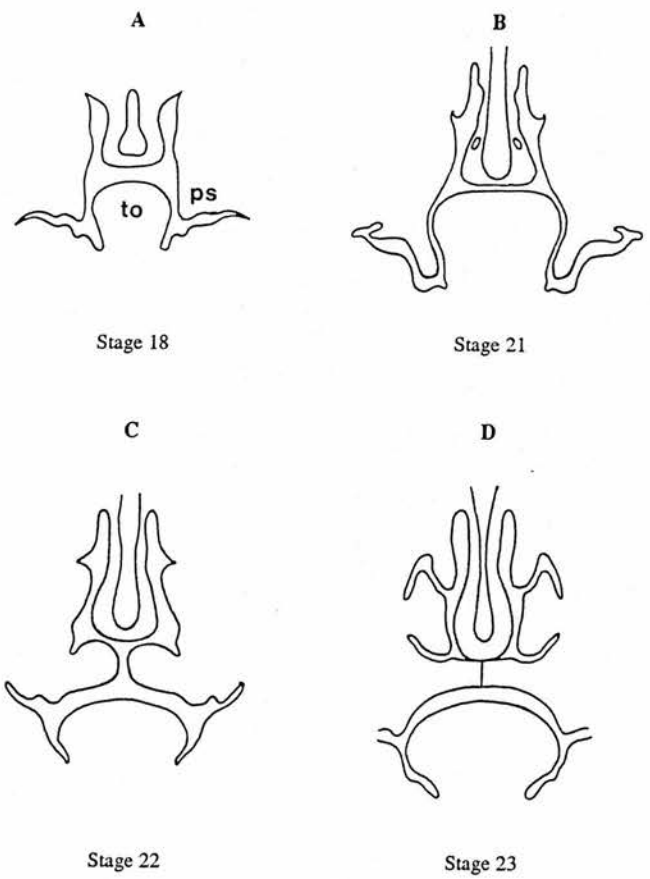


FIGURE 2. Normal stages of mammalian palatogenesis. Diagrammatic representation of the normal stages of mammalian palatogenesis. (A) At Stage 18 the palatal shelves (ps) become visible as ridges on the maxillary processes. (B) The palatal shelves grow vertically down the side of the tongue (to), reaching their full length by Stage 21. (C) In Stage 22 reorientation of the palatal shelves brings them into apposition above the tongue. (D) The palatal shelves fuse in the midline in Stage 23

shelves to a bilayer with flattened cells covering a layer of irregularly shaped cells in early palatal shelves. It would seem likely that the initiation of palatal shelf growth, at least in part, is controlled by epitheliomesenchymal tissue interaction, although the physiochemical nature of these signals is unknown. Drugs known to interfere with the early periods of palate development *in vitro* (6-mercaptopurine & Hadacidin) are inhibitors of nucleic acid synthesis (Shah 1984) and are, therefore, likely to act in a non-specific way. Other studies of initial palatal shelf growth using indicators of DNA synthesis have shown two periods of rapid mesenchymal proliferation one during initial shelf outgrowth and the other during the period immediately prior to shelf elevation (Burdett *et al.* 1988).

With rapid vertical growth, the shelves achieve their full length prior to elevation. This growth on either side of the tongue is the result of proliferation of mesenchyme (Fig.2B) (Ferguson, 1987). It is often stated that the palatal shelf mesenchyme is of cranial neural crest origin (Greene & Pratt 1976; Ferguson 1987) there is, however, no direct evidence for this in mammalian species. The cranial neural crest does, however, contribute significantly to the cell population of chick maxillary processes (Johnston & Listgarten, 1972) and several cranial neural crest-derived ectomesenchymal phenotypes appear during human palatogenesis (Slavkin, 1984).

1.3.2 Mechanisms of shelf elevation

In the stage 20 embryo a remarkable reorientation of the palatal shelves occurs, as both shelves rapidly elevate they come into apposition above the tongue. The origin of the intrinsic force of elevation has been the subject of much debate and is almost certainly multifactorial (Ferguson, 1987). Coleman (1965), using rat embryos, and Brinkley and Morris-Wiman (1984), using mouse embryos, demonstrated regional differences in the character of reorientation with the anterior densely-packed mesenchyme showing a rapid, all-or-nothing, rotational elevation while the posterior regions display a more fluid remodelling.

Zimmerman and Wee (1984) have suggested that contractile abilities of shelf

mesenchymal cells may participate in elevation. Contractile proteins, actin and myosin, have been localised to cytoplasmic, nonmuscle contractile systems on the oral and nasal sides of the murine vertical palatal shelves (Lessard *et al.* 1974; Kuhn *et al.* 1980) In mouse embryos, incubation of pre-elevation palatal shelves with adenosine triphosphate (ATP) causes condensation of cytoplasmic actomycin complexes which results in almost complete elevation of the anterior palate (Zimmerman & Wee 1984). The ability of certain neurotransmitters to stimulate (serotonin and acetylcholine) and inhibit (gamma-aminobutyric acid) shelf elevation in cultured murine palates is thought to be mediated *via* mesenchymal cell contractility and matrix degradation (Zimmerman & Wee 1984; Ferguson, 1988).

A role for the extracellular matrix (ECM) and, in particular, glycosaminoglycans (GAGs) in shelf elevation was first suggested by Walker and Fraser (1956). Pratt *et al.* (1973) studied the composition of the GAGs present in murine palatal shelves and found the major component (60-65%) to be hyaluronic acid (HA). The striking hydrophilic properties of HA has led to the suggestion that the large changes in osmotic pressure that result from a small increase in the concentration of HA (Comper & Laurent, 1978) may induce the rapid shelf movement in reorientation by expansion of a gel matrix. Treating mouse embryos with compounds that cause degradation of HA result in embryos with normal elevation of the anterior portion of the palate and defective reorientation posteriorly (Brinkley, 1980).

Brinkley and Morris-Wiman (1984) also studied the role of the shelf epithelia in directing the intrinsic force. Using [^{3}H]thymidine derived mitotic indices, they identified areas of morphogenetic activity on the superior nasal and mid-oral epithelial surfaces of murine palatal shelves, it is not clear whether the latter areas differ from the developing palatal rugae. The function of the rugae that develop on the oral surface of the palate is not known. Recent studies have shown a close temporal relationship between development of the rugae and the secondary palate and suggest a possible role in elevation (Sakamoto *et al.* 1989).

It has thus been proposed that at least two regionally distinct mechanisms are

involved in mammalian shelf elevation. Anteriorly, an intrinsic system of contractile proteins cause a rapid reorientation. Posteriorly expansion of a mesenchymal gel matrix produces a force which is then directed by local proliferative changes in the oral epithelial component and bundles of type I collagen (Ferguson, 1987) which produces a more fluid reorientation.

Many other factors have been proposed as having a role in shelf elevation these include; the mechanical effect of substantial vertical head growth with little lateral growth (Diewert 1978), straightening of the flexed cartilaginous, cranial base (Taylor & Harris, 1973) and pressure changes produced by foetal mouth opening (Humphrey 1969), swallowing movements (Walker 1969) and hiccuping (Ferguson 1988). Although these have generally been elegant and detailed studies, supporting evidence is lacking.

1.3.3 Fusion of the shelves

Almost immediately after elevation the medial edge epithelia (MEE) of both shelves come into contact above the tongue. In the mouse embryo, fusion begins at the border of the anterior and middle thirds of the secondary palate in the region of the second rugae (Sakamoto *et al.* 1989) with formation of the midline epithelial seam (MES) and proceeds in both anterior and posterior directions until completion by stage 23. The final process of fusion involves disruption of the MES to establish mesenchymal continuity.

Prior to elevation the MEE resembles other epithelia lining the oronasal cavity. In hamster embryos the MEE is a bilayer consisting of a surface squamous layer (periderm) overlying a glycogen-rich cuboidal cell layer (Chaudhry & Shah, 1973) in contact with an intact basal lamina. The cells are connected both within and between layers, by numerous small desmosomes. After elevation, but prior to midline contact, the cells in the peridermal layer of the MEE loose their intercellular connections, become irregularly shaped and are shed from the outer surface (Waterman & Meller, 1974). In mouse embryos, the basal layer of the MEE shows continued heavy staining for glycogen, abundant rough endoplasmic reticulum (RER) and occasional mitotic figures (Fitchett & Hay, 1989).

As the MEE fuse to form the MES the mechanism of initial adherence is thought to be the formation of glycoconjugates on 'sticky' cell surfaces (Pratt, 1983; Shah, 1984). This 'stickiness' is, however, specific in mouse embryos, as the MEE will only adhere to one another and not to other oral epithelial surfaces (Ferguson *et al.* 1984). None of the known cell adhesion molecules show specific staining in murine palatal epithelia (Obrink 1986), although certain carbohydrate rich-molecules (Con A & *Ulex europaeus*) have been localised to the MEE (Ferguson, 1988). In human embryos the MES proper is established by the formation of desmosomes between adhering cells (Morgan & Pratt, 1977; Shah, 1984). Ferguson (1988) has shown desmoplakin accumulation (a desmosomal plaque protein) in murine MEE, suggesting that there is specific and rapid construction of desmosomal components immediately prior to fusion.

1.3.4 Disruption of the midline epithelial seam

Disruption of the MES has been cited as a prime example of programmed cell death (Saunders 1966; Green & Pratt 1976). Evidence for this was the apparent cessation of DNA synthesis in the MEE of rat embryos 24-36 hours prior to fusion (Hudson & Shapiro, 1972; Pratt & Martin, 1975), reports of macrophages and lysosomes in the rat MES *in vitro* (Hayward, 1969) and an increase in cyclic adenosine monophosphate (cAMP), which is known to induce MEE cell death *in vitro* (Pratt & Martin, 1975), in the prefusion murine palatal mesenchyme. However Fitchett and Hay (1989) convincingly showed that after sloughing of the periderm layer, cell death is uncommon in the immediately prefusion MEE or the MES in the rat embryo. They also presented evidence for epithelial-to-mesenchymal transformation within the MES with characteristic changes in cell morphology (prominent nucleoli, 'robust' nuclei) and alteration in the cytoskeletal profile from predominantly keratin (epithelial) to vimentin (mesenchymal). They propose epithelial-to-mesenchymal transformation as the primary mechanism of MES disruption.

Dramatic changes in morphology of the remaining mammalian palatal epithelia occur immediately after the disruption of the MES. The nasal epithelium differentiates into pseudostratified ciliated columnar cells and the

oral epithelium into stratified squamous cells (Sharpe & Ferguson 1988). Using elegant epithelial-mesenchymal recombination techniques Ferguson & Honig (1984) have shown that epithelial differentiation in the palatal shelves, both within and across species, is controlled by the underlying mesenchyme.

In an attempt to identify the mesenchymal signals involved in epithelial differentiation the distribution of extracellular matrix (ECM) molecules in the murine palate around the time of fusion have been extensively studied using immunohistochemical methods (Ferguson, 1988). The ECM may be involved in the signalling process either directly or *via* interactions with soluble factors such as growth factors. Most ECM molecules studied during palate development were either undetectable (collagen types II, VIII and X) or appeared to be ubiquitously distributed (collagen types I and III and fibronectin). However several molecules did show specific staining patterns. Collagen type IV and laminin were found throughout the epithelial basement membrane while collagen types V and VII were localised to the MEE basement membrane. Tenascin and collagen type IX have the most interesting distribution patterns with both localised temporally and spatially around the time of MES disruption. Collagen type IX has been localised to the intersections of collagens in other systems (Muller-Glauser *et al.* 1986), although its role during palate fusion remains unclear. More is known of the *in vitro* and *in vivo* properties of tenacin and its close relationship with transforming growth factor type beta expression which will be discussed in section 1.5.

1.3.5 The post-fusion palate

There are very few studies of the ultrastructural or biochemical processes involved in mesenchymal differentiation after disruption of the MES. It is known that the subsequent differentiation of the ectomesenchyme must be regionally-specified as the anterior portion (hard palate) undergoes membranous ossification and the mesenchyme of the posterior palate differentiate to myoblasts and form the musculature of the soft palate, (tensor veli palatini, musculus uvulae etc.), the nature of the physiochemical signals determining this polarity is, however, unknown (Slavkin, 1984).

1.4 GROWTH FACTORS IN PALATE DEVELOPMENT

With increasing knowledge of their *in vitro* properties, peptide growth factors have been cited as candidate genes for study of the complex signalling systems that coordinate division, differentiation and cell death during palate development (Ferguson 1988). Evidence for their involvment in other morphogenetic processes is provided by studies of both RNA and protein localisation that show spatial and temporal patterns of peptide growth factors and their receptors in mammalian embryos (Heine *et al.* 1987, Lehnert and Akhurst 1988, Pelton *et al.* 1989). Many of these growth factor genes also display extraordinary degrees of evolutionary conservation, have sequence and structural similarities to developmental genes and exhibit *in vitro* properties suggestive of a role in development (Wozney *et al.* 1988, Seyedin *et al.* 1986; Padgett *et al.* 1987, Weeks and Melton 1987, Greenwald *et al.* 1985, Wharton *et al.* 1985; Slack *et al.*1987, Kimelman and Kirschner 1987). Five of the major classes of peptide growth factors will be dealt with in more detail below, although many other peptide growth factor families exist (eg. nerve growth factors, cytokines, colony stimulating factors and interleukins) they have not been cited as candidate genes in the study of palatal development (Ferguson, 1988).

1.4.1 Insulin-Like Growth Factors

There are two isoforms of insulin-like growth factor, IGF-I and IGF-II, both are single chain polypeptides with extensive sequence and structural homology to proinsulin. Although their physiological role is not yet clear, both are potent promoters of growth and differentiation in many cell types *in vitro* (Froesch *et al.* 1985). Studies of IGF expression patterns in development using in situ hybridisation to sectioned rat embryos have demonstrated expression of IGF-I in the somitic, hepatic bud and branchial arch mesoderm and IGF-II in myoblast, maturing cartilage and centres of membranous ossification (Beck *et al.* 1987). No specific hybridisation to palatal tissues was detected.

1.4.2 Heparin-binding Growth Factor Family

The prototypes of this family, acidic and basic fibroblast growth factors (aFGF and bFGF) were first isolated from bovine neural tissue on the basis of their mitogenic effect on a variety of cells of mesenchymal origin (reviewed by Lobb *et al.*, 1986). Recently new family members have been identified: int-2 (Dickson & Peters, 1987), kFGF (Taira *et al.* 1987), FGF5 (Zhan *et al.* 1988) and FGF6 (Marics *et al.* 1989). All members of the family display 40-50% amino acid identity with each other.

Although the physiological role of this family would appear to be angiogenesis (Folkman & Klagsbrun, 1987), recently a role in mammalian development has been proposed following studies that implicated both int-2 (Wilkinson *et al.* 1988) and FGFs (Slack *et al.* 1987) in the process of mesoderm induction in the early embryo. Although no detailed embryonic *in situ* localisation of these molecules has been published as yet, *in vitro* studies on cultured cells from the medial edge palatal mesenchyme (MEPM) have shown that both aFGF and bFGF induce profound changes in cell morphology, stimulate GAG production and have a potent mitogenic effect (Sharpe & Ferguson, 1988). The role that these substances have in the developing palate is unclear, although the authors have suggested that they may have a role in shelf cell migration.

1.4.3 Platelet-derived growth factor family

Platelet-derived growth factor (PDGF) is the principal mitogen present in whole blood serum, stimulating the growth of connective tissue derived cells, thus suggesting that PDGF may have an important physiological role in the wound healing process (Ross *et al.* 1986). Two distinct chains are synthesised (A and B chains) and these are thought to form both homo- and heterodimers. Transcripts of both these chains have been discovered in stage 6 mouse embryos (Mercola & Stiles, 1988) suggesting an important role in morphogenetic events. The only clue that PDGF may have a role in palatogenesis comes from their reported mitogenic effect on cultured MEPM cells, although unlike FGF, EGF and TGFß family members it does not seem

to alter the ECM profile *in vitro* (Sharpe & Ferguson 1988)

1.4.4 Epidermal Growth Factor Family

This group of peptide growth factors have been studied more than any other in palatogenesis, this is largely due to the pioneering work of Robert Pratt and his co-workers in Bethesda. Although much of the *in vitro* work has used epidermal growth factor (EGF), it would now appear that transforming growth factor alpha (TGF alpha) is the predominant molecule of this group interacting with the EGF receptors during mammalian development (Adamson *et al.* 1981). TGF alpha has, therefore, been termed the embryonic homologue of EGF (Derynck, 1985). The two molecules share 30% amino acid sequence homology and are interchangeable in a number of biological assays (Derynck, 1988).

EGF has been shown to have remarkable effects on palate epithelial and mesenchymal cells in culture. Hassell (1975) and Tyler and Pratt (1980) have shown that the MEE of palates cultured in the presence of EGF express a nasal cell phenotype and terminal differentiation of the peridermal cells is inhibited, thus preventing the fusion process. In a follow-up study, it was found that cyclic adenosine monophosphate (cAMP) could partially block this effect of EGF (Hassell & Pratt, 1976). Later Grove and Pratt (1983) found that EGF was essential for the growth and differentiation of palatal epithelia cultured on plastic or extracellular matrix (ECM). EGF also can stimulate mesenchymal synthesis of type V collagen (Silver *et al.* 1984) and hyaluronic acid (Turley *et al.* 1985). Nexo *et al.* (1980) found a dramatic increase in the activity of an EGF-like substance (presumably TGF alpha) in mouse embryos around the time of palatogenesis.

The EGF receptor has also proven to be of great interest in the study of the developing palate as it has been localised to the epithelia of the vertical palatal shelves (Nexo *et al.* 1980). Yoneda and Pratt (1981) have shown that palatal mesenchymal cells also contain a high level of EGF receptor and that these cells are responsive to the growth promoting properties of EGF.

It would seem, therefore, that the EGF family of growth factors has an important role in growth and differentiation during palatogenesis. Ardinger *et al.* (1989) have provided further indirect evidence by showing an association between a restriction fragment length polymorphism detected by a TGF alpha gene probe in genomic DNA and in patients with isolated CL(P).

1.4.5 The transforming growth factor type-beta family

The TGFßs are multifunctional molecules with predominantly growth inhibitory or differentiative effects on a wide variety on cell types. TGFß -related factors have recently emerged as polypeptides with central roles in development. There are at least eight different types of TGFß (only three have been found in mammals) with many other structurally-related proteins are included in a 'superfamily'. The TGFß receptors would also appear to exist in several forms although little is known about the molecular nature of the receptor molecules and the intracellular signalling that results in the diverse effects of this group.

1.4.5.1 TGFß1

Transforming growth factor type beta 1 (TGFß1) was discovered independently by two groups (Moses *et al.* 1981; Roberts *et al.* 1981) through its ability to induce anchorage-independent growth of rat fibroblasts in soft-agar culture. The gene has been mapped to 19q13.1 in the human and produces a transcript of 2.5 Kb (Sandberg *et al.* 1988). The active (or mature) complex of TGFß1 is a dimer of identical 112a.a. polypeptides. This is synthesised as a large precursor polypeptide (390a.a.) that is proteolytically cleaved releasing a carboxyterminal mature monomer (112 a.a.) (Derynck *et al.* 1985). The amino acid sequence of the mature region displays an extraordinary degree of interspecies conservation (Derynck *et al.* 1986; Jakowlew *et al.* 1988). The precursor region displays much less homology between species (20-30%) and its role within the cell is not yet clear, although it appears to be necessary for the efficient secretion of the molecule (Sha *et al.*1989).

TGFß1 appears to leave the cell as a latent complex (Miyazono *et al.* 1988, Wakefield *et al.* 1988) consisting of the mature and precursor portions of the protein bound with a TGFß-binding protein (Miyazono, 1989). Activation occurs in the extracellular environment and can be achieved *in vitro* by transient acidification or limited proteolysis. As TGFß1 is synthesised by most cell types (Derynck *et al.* 1987) and its receptors are ubiquitously distributed (Wakefield *et al.* 1987), it would appear that the activation of the latent complex has a major role in the regulation of TGFß biological activity.

1.4.5.2 TGFß2

TGFß2 was independently isolated by four groups (Seyedin *et al.* 1987; Cheifetz *et al.* 1987; Hanks *et al.* 1988; Wrann *et al.* 1987), it is located on 1q41 in the human. The TGFß2 precursor protein is 442a.a. with similar post-translational processing to TGFß1, producing a mature protein of 112a.a.(Madisen *et al.* 1988). There is 71% amino acid sequence identity with TGFß1 including conservation of all nine cysteine residues. The TGFß2 mature peptide portion also shows remarkable cross species conservation (De Martin *et al.* 1987; Madisen *et al.* 1988; Hanks *et al.* 1988; Cheifetz *et al.* 1987; Seyedin *et al.* 1987).

1.4.5.3 TGFß3

TGFß3 has been cloned by several groups screening human, porcine, murine and chick cDNA libraries with TGFß1 probes (ten Dijke *et al.* 1988, Miller *et al.* 1988; Jakowlew *et al.* 1988a; Kondaiah *et al.* 1990). The gene has been localised to 14q24 in the human. It is synthesised as a 412a.a. peptide with the C terminal 112 amino acids sharing approximately 80% amino acid identity with TGFß1 and TGFß2 and has a high level of cross species sequence identity (ten Dijke *et al.* 1988a; Derynck *et al.* 1988; Jakowlew *et al.* 1988a). TGFß3 is the most abundant TGFß family member expressed in chick embryos and might, in this organism, fulfil the function of TGFß1. There is little information on the biological activity or localisation

of TGFß3 in mammalian systems. Recently, however, ten Dijke *et al.* (1990) have shown that recombinant TGFß3 is three to five times more potent than TGFß1 in certain biological systems.

1.4.5.4 Other TGFßs and the superfamily

TGFß4 was cloned from a chick library (Jakowlew *et al.* 1988b) it is unusual in that the precursor only contains one N-glycosylation site (Jakowlew *et al.* 1988b) which suggests that TGFß4 protein may not be secreted or is secreted by the cell using a different mechanism. The mature portion of the TGFß4 peptide is also unusual in that it is 114a.a. with a 2 amino acid insertion near the N-terminus. TGFß5 was cloned from a *Xenopus* library (Kondaiah *et al.* 1990) and is expressed throughout amphibian early development and in the *Xenopus* XTC cell line (Kondaiah *et al.* 1990). These new forms of TGFß's do not represent the chicken or *Xenopus* homologues of TGFß1, since the chicken TGFß1 cDNA has been cloned and the predicted mature protein found to be 100% identical to human TGFß1 (Jakowlew *et al.* 1988). Recently Thomsen and Melton (1990) have cloned 8 new members of the family in the *Xenopus*, no further details of these are available as yet.

The members the TGFß superfamily include well characterised proteins such as the inhibins and activins. They show between 22% and 38% amino acid sequence identity with TGFß and all share at least seven of the nine C-terminal cysteine residues. They are synthesised as large precursors with carboxyterminal mature sequences with many members of the superfamily implicated in the regulation of developmental processes (see Table IV).

1.5 THE BIOLOGICAL PROPERTIES OF TGFß

Most of the work described in this section relates to studies of the *in vitro* properties of TGFß1 peptide, however, much of the work has been repeated using TGFß2 which has *in vitro* properties almost identical to those

TABLE IV Members of the TGFß superfamily

Substance	*Homology with hTGFβ1*	*Reference*	*Developmental role*
Müllerian inhibiting substance (MIS)	31%	Cate et. al. 1986	Causes regression of the Müllerian duct during the development of the reproductive tract in male embryos
Inhibin	alpha 46% beta 38%	Mason et. al.1986	Inhibits the secretion of follicle stimulating hormone (FSH) from the pituitary
Decapentaplegic (DPP)	36%	Padgett et. al. 1987	Determination of the dorso-ventral axis in the early embryo and correct morphogensis of the imaginal disks in the drosophila larva.
Vg1	36%	Weeks & Melton 1987	Localized in the cells which produce the mesoderm inducing signal in the frog embryo.
XTC-MIF	?	Rosa et. al. 1988	Mesoderm-inducing factor
Vgr-1	34%	Lyons et. al. 1989	Vg-related protein
BMPs	BMP-2 38% BMP-3 34%	Wozney et. al. 1988	Bone morphogenetic proteins

of TGFß1 (Seyedin *et al.* 1987, Cheifetz *et al.* 1987). In some *in vitro* systems TGFß1 and TGFß2 have been shown to form heterodimers (Cheifetz *et al.* 1987; Danielpour *et al.* 1989) these have, however, not been detected *in vivo*. One major difference between TGFß1 and TGFß2 is the ability of the latter to induce mesoderm formation in *Xenopus* embryos (Rosa *et al.* 1988).

1.5.1 The effect of TGFß on cell growth and differentiation

Although originally described as a mitogen, TGFß has proven to be growth inhibitory to most cell types in culture. This inhibition appears to be particularly evident in epithelial cell cultures, with TGFß showing reversible inhibition of cultured keratinocytes (Shipley *et al.* 1986, Wilkie et. al. 1988), but causing irreversible inhibition of proliferation in intestinal (Urokowa *et al.* 1987), bronchial (Masui *et al.* 1986) and tracheal epithelial cells (Jeyyen *et al.* 1986) with the appearance of terminal squamous differentiation markers.

TGFß also has dramatic effects on cells of the lymphatic system being one of the most potent immunosuppressives known, inhibiting lymphocyte proliferation at femtomolar concentrations (Kehrl *et al.* 1986; Ristow *et al.* 1986, Ellingsworth and Nguyen 1988) and reducing IgG secretion by B-cells (Kehrl *et al.* 1986b).

In contrast to the above examples endothelia display a paradoxical response to TGFß, with inhibition of FGF-induced proliferation of cultured endothelial cells (Frater-Schroder 1986) whereas the subcutaneous injection of TGFß evokes a strong angiogenetic response (Roberts *et al.* 1986). This *in vivo* response is almost certainly effected indirectly via TGFßs chemotactic action on monocytes (Wahl *et al.* 1987) and the release of angiogenetic factors from other cells.

The effect of TGFß on the cells of the liver has of considerable interest because of the regenerative abilities of this organ. TGFß inhibits EGF-induced growth of hepatocytes *in vitro* at very low concentrations

(Nakamura *et al.* 1986; Carr *et al.* 1986; McMahon *et al.* 1986) and localisation of TGFß RNA have implied a negative regulatory role, by paracrine mode of action, in the regenerating liver (Brann *et al.* 1988).

TGFß has also proven to be a complex regulator of cell differentiation as well as growth. It induces differentiation of certain cell types *in vitro* such as bronchial epithelium (Masui *et al.* 1986), chondrocytes (Seyedin *et al.* 1986) and keratinocytes (Reiss & Sartorelli, 1987) while inhibiting differentiation of adipogenic and myogenic cells (Ingotz & Massangue 1986) with prevention of myotube fusion and the expression of muscle specific proteins (Massague *et al.* 1986, Florini *et al.* 1986, Olson *et al.* 1986; Allen and Boxhorn 1987).

1.5.2 The effect of TGFß on the ECM

Induction of extracellular matrix synthesis is one of the most interesting properties of TGFß. This is achieved by three mechanisms. Firstly TGFß increases the rate of synthesis of the ECM molecules, collagen (type I, II & III), fibronectin (Ignotz & Massague 1986; Seyedin *et al.* 1985; Varga et. al. 1987), tenascin (Pearson *et al.* 1988), thrombospondin (Pentinnen et. al. 1988) and cartilage-specific proteoglycans (Seyedin *et al.* 1985). Although most work has been done on fibroblasts, tissue specific effects on ECM are seen in bone (Noda and Rodan 1987), endothelial cells (Madri et. al. 1988) and human arterial smooth muscle cells (Chen *et al.* 1987). Secondly, TGFß effects a decrease in synthesis of the protease enzymes; collagenase (Edwards *et al.* 1987), elastase (Redini *et al.* 1988), transin/stromelysin (Machida *et al.* 1988) and increases the transcription and synthesis of protease inhibitors such as plasminogen activator inhibitor (PAI, Lund *et al.* 1987) and tissue inhibitor of metalloproteases (Edwards *et al.* 1987). Thirdly, TGFß also modulates the interaction between mesenchymal cells and extracellular matrix by stimulating the expression of surface receptors for extracellular matrix molecules on fibroblasts *in vitro* (Ignotz and Massague 1987, Allen-Hoffmann *et al.* 1987). All three of these actions could effect the rapid build-up of ECM that is important in morphogenetic processes as well as wound healing (see 1.5.1.4).

1.5.3 TGFß in specific systems

1.5.3.1 Wound healing

The commercial and clinical implications of TGFßs' role in wound healing has made this the subject of much study since Roberts *et al.* (1986) showed that TGFß injected into neonatal mice produces a localised fibrotic response that is similar to a wounding response and Assoian and Sporn (1986) found TGFß to be released from platelets at sites of injury. It has recently been called a 'biological glue' after it was shown to induce choroidoretinal adhesions following experimentally induced retinal tears (Glaser at al. 1989). Monocyte chemotaxis, activation of growth factor production (Wahl *et al.* 1987), deactivation of macrophage hydrogen peroxide (Tsunawali *et al.* 1988) and blocking of the cytotoxic activity of natural killer cells (Rook *et al.* 1986) are all properties of TGFß that may be involved in the controlled tissue response to damage.

1.5.3.2 Bone formation and repair

Adult bone is the richest source of TGFß in the body (Seyedin *et al.* 1987). TGFß also seem to be important in morphogenesis of the skeletal system as it is synthesised in fetal bone cells (Heine *et al.* 1987; Sandberg et. al. 1988). The *in vitro* effects of TGFß on the cells of the developing skeletal system are complex with site and stage specific effects on ECM synthesis (Rosier *et al.* 1988) and chondroblasts and chondrocytes growth *in vitro* (O'Keefe *et al.* 1988). The role that TGFß plays in the homeostasis and repair of adult bone is less clear, although it may be involved in bone resorption as part of the constant remodelling (Tashijian *et al.* 1985) and has been localised in the callous of healing fractures (Borlander *et al.* 1989).

1.5.4 TGFßs in embryogenesis

Most of the work that suggests a role for the TGFßs as embryonic

modulators comes from localisation of either protein or RNA in sectioned mouse embryos. Both TGFß1 and TGFß2 are known to be synthesised in the preimplantation mouse embryo (Rizzino 1985; Mummery *et al.* 1990) although their role is not yet clear. Akhurst *et al.* (1990) found high concentrations of TGFß1 RNA in the extraembryonic blood islands suggesting a role for this gene in both haematopoiesis and angiogenesis. One major role for TGFß isoforms in the embryo would appear to be in epithelial mesenchymal interactions. Lenhert and Akhurst (1988) showed TGFß1 RNA to be localised to epithelia overlying mesenchyme where the protein had been localised (Heine *et al.* 1987) such as the tooth bud and whisker follicle thus suggesting a paracrine mode of action in these systems. TGFß isoforms has also shown site- and stage- specific RNA localisation patterns during cardiac development (Akhurst *et al.* 1990), lung and gut development (Pelton *et al.*1989; Gatherer *et al.* 1990; Millan *et al.* 1990)(see section 4.2).

1.5.4.1 TGFß in palate development

The ability of TGFß to alter the ECM profile and to control mesenchymal growth and induce terminal differentiation in certain epithelia make it of great interest in the study of palatogenesis. Heine *et al.* (1987) used immunohistochemical techniques to detect TGFß in the mesenchyme of the developing palate. Subsequently Ferguson *et al.* (1988) have shown TGFß can inhibit the growth of cultured palatal mesechyme, stimulate synthesis of fibronectin and collagen types III, IV and V and inhibit collagen I synthesis (see section 4.2).

1.6 AIMS OF THE STUDY

The aims of this study fall into two broad groups:

1. a) Calculation of the birth prevalence of cleft lip, cleft palate or both by attempting complete ascertainment of all cases born to

mothers resident in the West of Scotland during a five year period beginning 01/01/80.

b) Use of the collected data to investigate laterality, sex ratio, seasonal and geographical distribution within the cleft lip group (with or without cleft palate) and the cleft palate group.

c) Identification of those patients in the group that have recognised diagnoses and tabulation of associated abnormalities in the 'unknown' group.

d) Establishment of a database of these patients that can be used in future studies of empiric recurrence rates and association studies.

2. a) To make staged mouse embryo sections of the critical stages of murine palatogenesis and, using *in situ* hybridisation of riboprobes to these sections, identify the expression patterns of genes encoding for transforming growth factor to type beta type 1 (TGFß1), TGFß2 and TGFß3 during murine palatogenesis.

b) To make staged mouse embryo sections from mice treated with retinoic acid at dosage and developmental stage that is known to cause clefting of the palate and investigate the effect of retinoic acid treatment on the expression patterns of genes for TGFß1, TGFß2 and TGFß3 in these embryos.

CHAPTER 2 MATERIALS AND METHODS

2.1 BIRTH PREVALENCE STUDY

The birth prevalence study was conducted over a five year period from the 1st January 1980 to 31st December 1984. The births studied were those to mothers whose first residence was within Greater Glasgow, Lanarkshire, Forth Valley, Dumfries & Galloway, Argyll & Clyde or Ayrshire & Arran Health Boards. The figures for both the number of total births and live births over this period for residents of these Health Boards, were kindly supplied by Dr. Susan Cole, Common Services Agency, Trinity House, Edinburgh. These figures were then subdivided by month of birth and sex of child to facilitate comparison with the ascertained group. A calculation of birth prevalence was used in preference to an estimation of the incidence of facial clefting as the common occurrence of facial clefts in early spontaneous abortions (Iizuka, 1973), would make the latter figure impossible to calculate.

2.1.1 Ascertainment

The index cases were identified through four main sources a) SMR11 neonatal discharge forms b) the Glasgow EUROCAT register c) hospital diagnostic indices of the Royal Hospital for Sick Children, Glasgow, Cannisburn Hospital, Glasgow and Seafield Hospital, Ayr d) SMR1 hospital admission records, kindly supplied by Dr. John Clarke, Common Services Agency, Trinity House, Edinburgh. Follow-up information on the index cases with associated abnormalities was obtained from hospital records, West of Scotland regional genetic records and detailed post-mortem reports. Both ascertainment and follow-up were aided considerably by the fact that all facial cleft patients that come to operation in the West of Scotland were treated by two regional specialist surgeons who have kept independent records of the patients they have seen and treated. Patients with microforms of facial clefts (bifid uvula and submucous cleft palate) were excluded as these defects may not come to medical attention and would, therefore, require a population survey for complete ascertainment.

2.1.2 Data Storage

The data recorded on each case were identifying number, name, date of birth, sex, residence post-code at time of birth, present address, details of facial cleft (type, side and severity), the number and nature of any associated abnormalities and whether a syndrome could be identified. The severity of the cleft was quantified using the method described by Jensen et al. (1988)(Fig.3). These data were stored on a microcomputer using the dBASE III plus program (Ashton-Tate).

2.1.3 Statistical Analyses

The birth prevalence figures were calculated per 1000 total births or live births and quoted with 95% confidence intervals. The standard deviations were calculated using the formula;

$SD = 1000 \times (N)^{-2}/n$ Czeizel & Tusnady (1984).

N = number of affected births
n = number of births in study period

To estimate the birth prevalence of facial clefts as isolated congenital anomalies, the number of cases in the study with no associated congenital anomalies was calculated (p_i). This figure however may be lower than the true figure for isolated CL(P) and CP as it ignores the possibility of random associations with other common congenital anomalies. To estimate a corrected birth prevalence for isolated facial clefts (p_c) the following calculation was used;

$p_c = 1000 \times p_i/1 - p_T$ Czeizel & Tusnady (1984)

p_T = total birth prevalence of congenital anomalies

The total birth prevalence for congenital anomalies of 36.6 per 1000 total

FIGURE 3

JENSEN SEVERITY SCORING SYSTEM

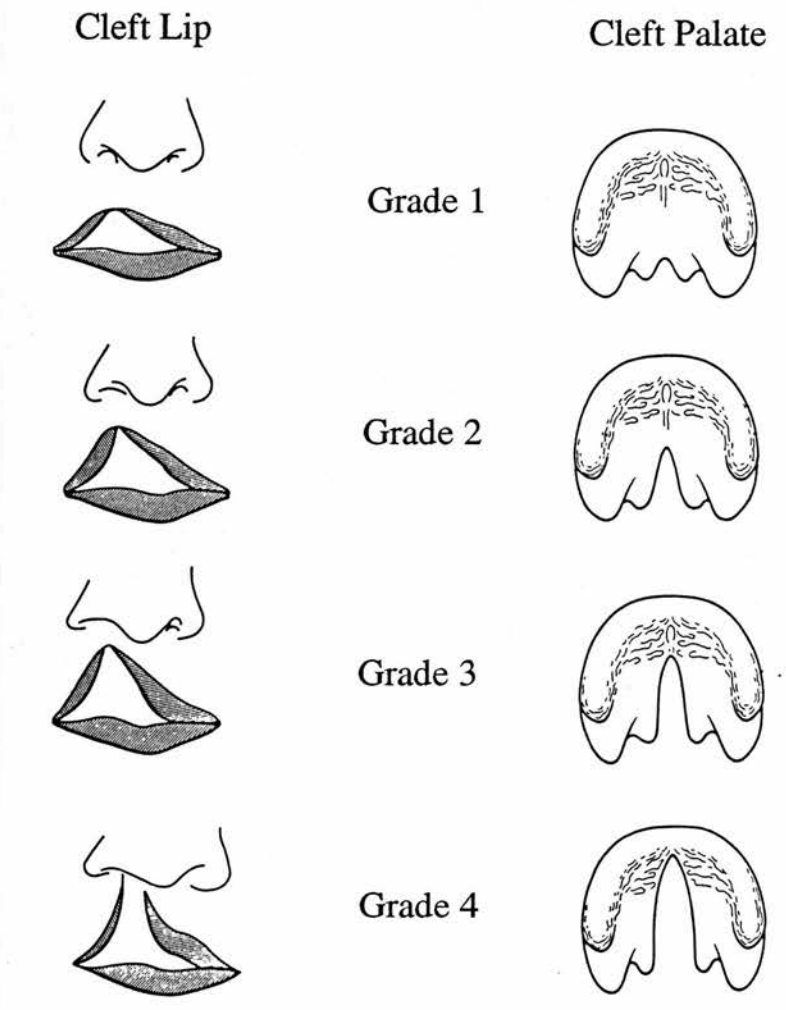


FIGURE 3. Jenson severity score. Diagrammatic representation of the scoring system used by Jensen et al. (1988). Cleft lip 1: notching of the upper lip to one-third of lip height 2: cleft of one- to two- thirds of lip height 3: two-thirds to subtotal cleft 4: total. Cleft palate 1: soft palate 2: one-third of hard palate 3:>one-third to subtotal 4: total

birth was used in the study was calculated from the Glasgow EUROCAT registery over the study period (De Wals & Lechat 1986).

Groups were compared using the chi-squared test, the Yates correction factor was used in the two by two contingency tables. Evidence of cyclical trends within the facial clefts groups were assessed using both the non-parametric rank-sum method described by Hewitt et al. (1971) and the parametric method described by Edwards (1961).

2.1.4 Syndrome identification

Many of the patients had been assessed prior to the study by one or other of the regional specialists involved in the care of facial clefts (Mr. P.A. Raine, consultant paediatric surgeon, RHSC, Glasgow and Mr. J.O. Boorman, consultant plastic surgeon, Canniesburn Hospital, Glasgow). The cases notes, post-mortem reports or obstetric records of all the index cases were reviewed. Syndrome diagnoses made prior to the study were critically evaluated, whenever possible clinical examination was carried out by the investigator; when this was not possible hospital or post-mortem records and clinical photographs were reviewed. All cases with associated abnormalities were assigned to 'known' (syndrome identified) or 'unknown' (no syndrome diagnosis) catagories. The unknown catagory was further reviewed with the help of Dr.J.L.Tolmie, consultant clinical geneticist, the London Dysmorphology Database and the Dysmorphology Group, Institute of Child Health, London.

2.2 RNA LOCALISATION STUDIES

The source of the chemicals and enzymes used in the study are shown in Table V.

2.2.1 Mouse Strains and Embryo Collections

In the first part of the RNA localisation study, outbred strains of NIH and

TABLE V

Sources of chemicals and enzymes

Chemical	*Source*
All-trans retinoic acid	Sigma
^{35}S uridine triphosphate	Amersham
BSA, DNAse and RNAse free	Pharmacia
Corn oil (pfs)	Sigma
Decon 90	Decon Labs.
Deoxyribonucleotides	Pharmacia
Dextran sulphate	Pharmacia
Diethylpyrocarbonate (DEPC)	Sigma
Dithiothreitol (DTT)	Sigma
EDTA	BDH
Ethidium bromide	Sigma
Formamide	Fluka
Histoclear	National Diagnostics
K5 nuclear track emulsion	Ilford
Molecular size markers (1kb ladder)	Gibco BRL
Panatomic X film	Kodak
Paraffin wax	BDH
Paraformaldehyde	Sigma
Phenol	Rathburn
Phenisol Developer	Ilford
Polyethylene glycol 800	Sigma
Proteinase K	Boehringer
Ribonucleotides	Pharmacia
Ribosomal RNA	Boehringer
7X detergent	Sterilin
Sephadex G50	Pharmacia
Spermidine	Sigma
TEMED	BDH
TESPA	Sigma
tRNA	Boehringer
Triethanolamine	Sigma
Tris base	Sigma
UTP-S	New England Nuclear
Enzyme	*Source*
DNAse I (RNAse-free)	Pharmacia
Klenow fragment of DNA polymerase I	Northumbria Biologicals
Proteinase K	Boehringer
RNA guard	Pharmacia
RNAse	Sigma
SP6 RNA polymerase (cloned, FPLC pure)	Pharmacia
T7 RNA polymerase (cloned, FPLC pure)	Pharmacia
T3 RNA polymerase (cloned FPLC pure)	Pharmacia

Parkes mice were used. Parkes females or NIH/Parkes females were mated with NIH males to generate litters and embryos. These animals were obtained from the National Institute of Medical Research, London. The C57BL/6 mice (Harlan Olac) used to generate the control and RA-treated embryos used in the second part of the study chosen for two reasons. (1) TGFβ expression in normal embryos of a different mouse strain could be studied thus showing whether expression patterns in NIH/Parkes embryos are strain-specific. (2) The effects of RA-administration (at different gestational ages and dosages) on palate development had been extensively studied in this strain (Abbott *et al.* 1989) thus facilitating experimental design.

The animals were kept on a 15 hr light/9 hr dark cycle. The day on which the copulation plug was found was called day 0.5 of development.

2.2.2 Dosing

All-trans retinoic acid (RA) was stored in the dark at -20^{0}C. The dosing solutions were prepared immediately prior to administration. RA was dissolved in 2 parts dimethyl sulphoxide and then 3 parts corn oil to a final concentration of 10mg/ml. Pregnant mice were weighed and received a single gavage of 100mg/kg on gestation day (GD) 12. Dosage and vehicle selection were based on the studies of Abbott et al. (1989). Embryos were collected on GD 13.5 and 14.5.

(Personal licence No: PIL 60/02854 issued under Animal (Scientific Proceedures) Act 1986, expires June 1991)

Other sections of RA-treated C57BL/6 murine embryos (100mg/Kg in corn oil on GD 10 & GD 12) were kindly supplied by Dr. Barbara Abbott (NIH, Bethesda). These sections, fixed and embedded using an identical protocol, were supplied as part of a collaborative study of the effect of RA administration on TGFß expression patterns

2.2.3 Preparation of solutions and glassware techniques

All solutions for RNA techniques, including solutions for the prehybridisation treatments of sections for *in situ* hybridisation and solutions used in riboprobe synthesis reactions, were millipore filtered, treated with diethylpyrocarbonate (DEPC) and autoclaved unless otherwise specified.

All glassware used in the above mentioned procedures was baked

overnight at 250^0C to destroy contaminating nucleases. Disposable plasticware was soaked in a DEPC solution and autoclaved for the same reason.

Chloroform/Isoamylalcohol

Chloroform was mixed with isoamylalcohol at 24:1 (v/v).

Denhardt's Solution (50x)

1% (w/v) bovine serum albumin, 1% (w/v) Ficoll and 1% (w/v) Polyvinylpyrolidone were dissolved in milliQ H_2O, cleared by millipore filtration and stored in aliquots at -20^0C.

Gel Loading Buffer (6x)

0.25% bromophenol blue and 0.25 % xylene cyanol were dissolved in 30 % glycerol in milliQ H_2O and stored at 4^0C.

Paraformaldehyde (PFA) solution

4% (w/v) paraformaldehyde was dissolved in phosphate buffered saline (PBS) at 70^oC and used the same day.

Phosphate Buffered Saline (PBS) pH 7.2

1 litre PBS was made up by dissolving 8g NaCl, 0.2 g KCl, 2.89 g Na_2HPO_4 (x12 H2O) and 0.2 g KH_2PO_4 in milliRO H_2O.

Phenol (pH5.0)

A 0.1% hydroxyquinolone solution in phenol was equilibrated with 0.3M

sodium acetate, pH 5.0 until the pH stabilised. Phenol pH 5.0 was stored at 4^0C protected from light under 0.3M sodium acetate, pH 5.0.

Phenol/Chloroform

Equilibrated phenol, chloroform and isoamyl alcohol were mixed at 25:24:1 and stored under buffer at 4^0C.

Polyacrylamide Stock

19% acrylamide (w/v) and 1% bisacrylamide (w/v) were dissolved in milliRO H_2O and stored at 4^0C in the dark.

Standard Sodium Citrate (SSC) pH 7.0

A 1 x solution of SSC is 0.15 M NaCl, 0.015M Na_3citrate. A 20 x concentrated stock solution was made by dissolving 175.3g NaCl and 88.2g sodium citrate in 1 litre of H_2O and adjusting the pH to 7.0 with NaOH.

TBE Electrophoresis Buffer (pH 8.3)

The working solution of this electrophoresis buffer is 0.089 M Tris-borate and 0.089 M boric acid. A 10 x concentrated stock solution was made by dissolving 121.0 g Tris base and 5.8 g of EDTA in 2 litres of H_2O and adjusting the pH to 8.3 by adding about 100g of solid boric acid.

2.2.4 Fixation and Embedding of Tissue

Tissue was fixed at $4^{O}C$ by mixing for 16-24 hrs in freshly prepared, ice-cold 4% PFA. The fixative was removed by rinsing with ice-cold PBS, then ice-cold 0.85% saline for 30 min each. The solution was then replaced with 10ml saline and an equal volume of 100% ethanol was layered on, followed by thorough mixing for 15 min. The specimens were then dehydrated by putting through ethanol:saline (1:1), two changes of 70% ethanol for 30 min each, 85% ethanol, 95% ethanol and 100 % ethanol for 30 min each. The ethanol was replaced by Histoclear for about 15 min or just before the specimen starts to darken. The specimens were then transferred to a prewarmed ($60^{0}C$) container of histoclear:paraffin solution (1:1) and incubated for about 15 min (at $60^{o}C$). The solution was replaced at $60^{0}C$ with filtered paraffin, three times for 20 min each. The specimens were transferred to a plastic mould, topped up with fresh paraffin wax, oriented as necessary with prewarmed forceps and the blocks cooled down quickly in an ice/water bath. Blocks were stored at $4^{0}C$.

2.2.5 Sectioning

3-Aminopropyltriethoxysilane (Tespa) coated slides were prepared according to the method of Rentrop et al. (1986). Microscope slides were washed overnight at $65^{0}C$ in 10% Decon and rinsed the next day in hot (approx. $60^{0}C$) running tap water for four hours. This was followed by 3 rinses in distilled water and a final rinse in milliQ water. The slides were baked overnight at $250^{0}C$ and dipped for 5 sec in 2% TESPA in acetone. The slides were then rinsed in four changes of acetone and four changes of milliQ water. This was followed by rapid, dust-free drying either in a 40-$50^{0}C$ oven or in a laminar flow hood. Slides were stored under dust-free conditions for up to two weeks before use.

Sections of 5-7 micron thickness were cut using a microtome with disposable blades. The sections were floated on a sectioning waterbath containing fresh milliQ water at $42^{0}C$ and dried on a slide-drier at $42^{0}C$

for 4 hours or o/n. If possible, the sections were used immediately or stored at 4^0C in the presence of dessicant.

2.2.6 Radiolabelled cRNA Probe Synthesis

To generate radiolabelled cRNA probes an *in vitro* transcription system was used (Fig.4). These riboprobes were produced with high (10^9 dpm/µg) specific activity, by using ^{35}S UTP (1000 Ci/mole) diluted 1:1 with unlabelled UTP-S. A typical incubation contained 75 pmoles each of radiolabelled and unlabelled S-modified UTP in a 10 µl reaction volume which also contained ATP, GTP and CTP at 1mM each and 1 µg of template DNA (see below) in the following buffer: 40 mM Tris pH 7.5, 6mM $MgCl_2$, 4mM spermidine, 10 mM DTT, 1µl RNAguard was added together with 10 units of SP6 polymerase and the incubation allowed to proceed for 60-90 minutes at 37^0C.

The transcription reaction was followed by DNase I digestion to remove the DNA template which might impair hybridisation reactions. RNase-free DNase I was diluted in 25 mM Tris pH 7.6, 50% glycerol to 500 units/ml and 6µl of this dilution used in a total reaction volume of 50µl containing 100mM NaOAc and 5mM $MgSO_4$. One more microlitre of RNAguard was added and the reaction incubated at 37^0C for 15 minutes. 30 µg carrier RNA (tRNA) was added before phenol extraction and ethanol precipitation.

To digest the probe down to a length of around 100 bases, which is considered the optimal probe length for *in situ* hybridization (Cox et. al. 1984), alkaline hydrolysis was performed. The precipitated RNA was taken up in 50µl of 10 mM DTT and a 1 x 10^6 dpm sample reserved for an analytical gel. 450µl of alkaline digestion buffer (100 mM $NaHCO_3/Na_2CO_3$ pH 10.2, DEPC treated and millipore filtered, DTT added to 10mM just before use) was added and the digestion was incubated at 60^0C for a length of time calculated by the following formula.

FIGURE 4

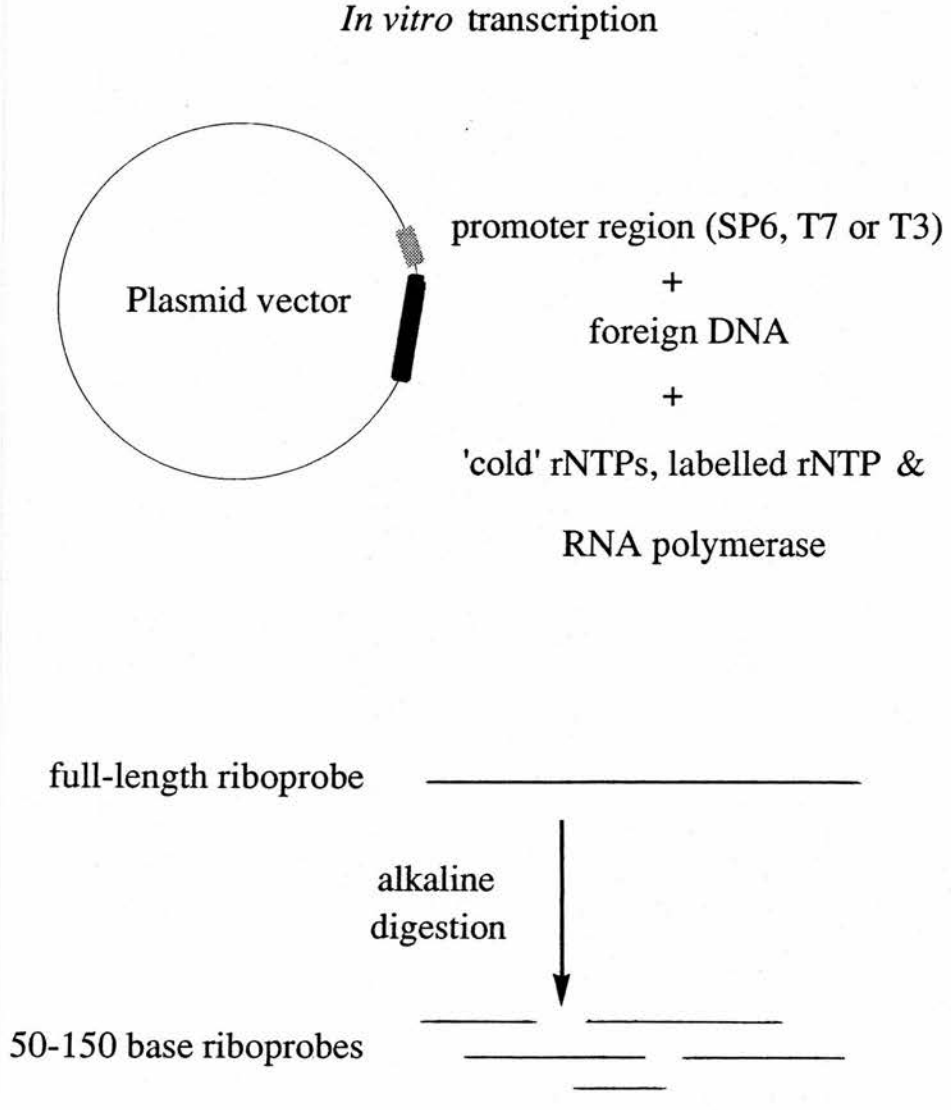


FIGURE 4. In vitro transcription system. Diagrammatic representation of the production of radiolabelled riboprobes by in vitro transcription method. A cDNA fragment of the gene to be studied is subcloned into a plasmid vector in specific orientation with respect to the RNA polymerase promotor. A mixture of labelled and unlabelled ribonucleotides can then be incubated with the RNA polymerase to produce a cRNA strand. This RNA is then fragmented by alkaline hydrolysis to produce probes that can be applied to tissue sections.

$$t = \frac{L_o - L_f}{k L_o L_f}$$

t = time in minutes
L_o = origi nal probe length in kb
L_f = desired probe length = 0.1
k = 0.11

For the full-length TGFß1 probe, incubation time was 90 minutes. The reaction was stopped by adding 50 ul 1M NaOAc pH 6.0, 50 ul 5% acetic acid and 10 µg carrier RNA.

The sample was then run over a Sephadex G50 spin column (1100 r.p.m/1 min.) to free it from the sodium carbonate salts and to eliminate any unincorporated radioactivity. The column buffer was 0.3 M NaOAc pH 5.0, 10 mM Tris, 1 mM EDTA, 0.1% SDS, with DTT added to 10mM just before use. The radiolabelled probe was precipitated with ethanol, vacuum dried and taken up in 10ul 10mM DTT. A 1 x 10^6 dpm aliquot of digest and undigested probe was run on a 6% polyacrylamide gel in 8M urea/TBE to check final probe length and quality of the original transcription product (Fig.5A-F).

2.2.6.1 TGFß1 Probe

The TGFß1-specific probe was a 600 nucleotide ApaI-KpnI fragment from the 5^{l} divergent precursor region of the gene (Fig.6A). This fragment was subcloned into Bluescribe (Stratagene) in antisense orientation with respect to the T3 promotor. The full-length murine TGFß1 cDNA (Derynck et al. 1986) was kindly provided by Dr R. Derynck (Genenteck). The subclone corresponds to the precursor region of the TGFß1 polypeptide (amino acids 68-268).

Figure 5

P.A.G.E. GEL OF RADIOLABELLED RIBOPROBES

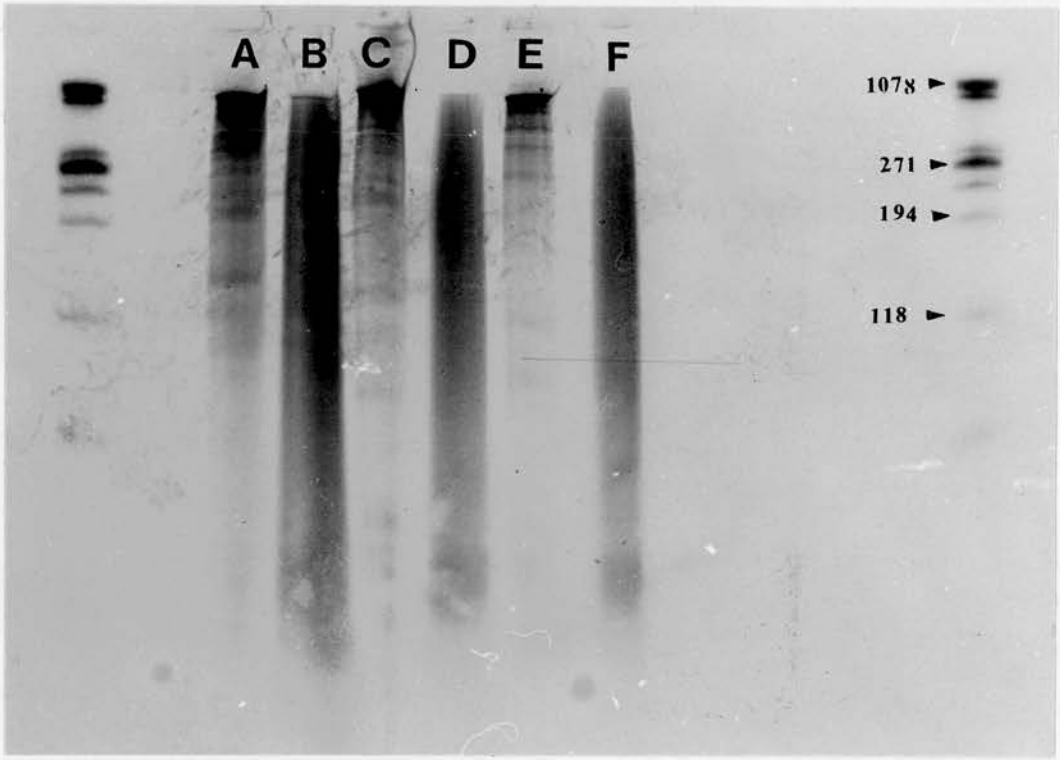


FIGURE 5. P.A.G.E. gel of radiolabelled riboprobes. Denaturing polyacrylamide gel of the radiolabelled cRNA produced by in vitro transcription, Full-length transcripts (A,C,E) and post-alkaline digestion products (B,D,F) are represented. (A,B) TGF beta1 (C,D) TGF beta2 (E,F) TGF beta3

2.2.6.2 The TGFß2 Probe

The TGFß2 DNA probe was obtained by amplification of the reverse transcriptase product of total mouse embryo RNA using the polymerase chain reaction (Saiki et al. 1988). The oligonucleotide primers spanned the initiation and termination codons. This probe was identical in nucleotide sequence to that reported by Miller et al. (1989) (F. Denhez unpublished). The initial experiments in this study used the full-length TGFß2 probe cloned into Bluescribe (Stratagene) in antisense orientation with respect to the T3 promotor. However in the latter part of the study a TGFß2 specific probe became available. This was a 501 nucleotide Pst1-Sac1 fragment, spanning amino acid residues 81-249 (Fig.6B), inserted antisense with respect to the T7 promotor. Both of these probes have shown identical hybridisation patterns during murine palatogenesis and in all other developmental systems in the mouse embryo (Millan *et al.* 1991)

2.2.6.3 The TGFß3 Probe

The TGFß3-specific probe was a 732 nucleotide Xba1 fragment subcloned into pBluescript KSII (Stratagene) orientated such that antisense probe was generated using the T3 promotor (Fig.6C). This region spans amino acid residues 8 to 251 of the precursor polypeptide (Denhez et al. 1990).

2.2.6.4 Control Probe

The control probe used was a full-length TGFß1 human cDNA, kindly supplied by Dr G. Bell (unpublished). It was also subcloned into the Bluescribe vector in sense orientation with respect to the T7 promoter.

Nucleotide sequence homologies between the three gene-specific probes were 42% (beta1 to beta2), 47% (beta2 to beta3) and 36% (beta1 to beta3)(Pelton et al. 1989; Denhez et al. 1990).

FIGURE 6

TGFß Gene Specific Probes

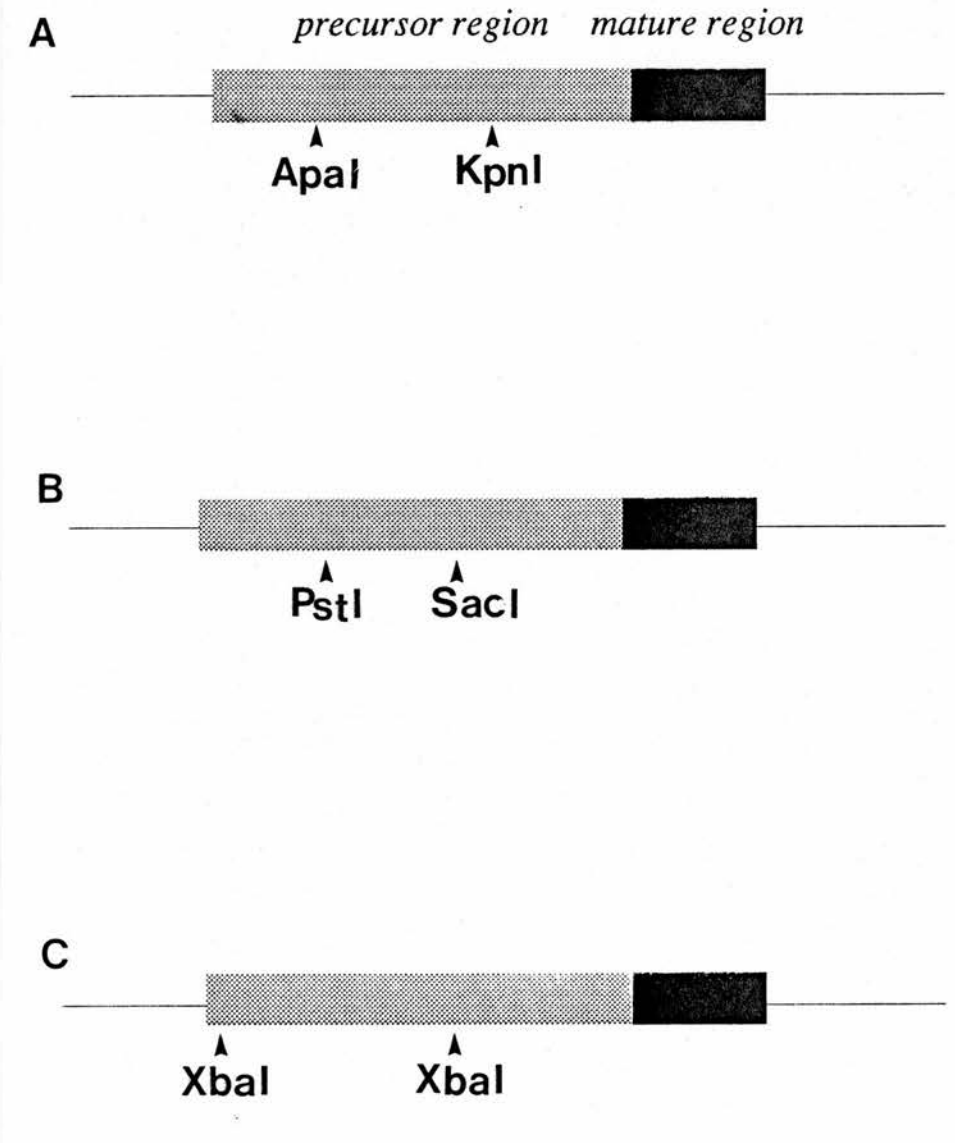


FIGURE 6. TGF beta subclones. Diagramatic representation of the cDNA fragments used to generate the riboprobes used in the study. (A) TGF beta1 ApaI-KpnI fragment (B) TGF beta2 specific PstI-SacI fragment (C) TGF beta3 XbaI fragment.

2.2.7 *In situ* Hybridization

The *in situ* hybridisation procedure used in the study is based on that of Wilkinson et al. (1987). Sections were de-waxed in fresh histoclear for 10 minutes (twice), washed in 100% ethanol for 2 minutes and rehydrated quickly by putting through an ethanol series (100%, 100%, 95%, 85%, 70%, 50%, and 30%) and rinsed in 0.85% saline, then PBS, for 5 minutes each. A 20 minute post-fixation in 4% PFA was followed by two 5 minute washes in PBS and treatment with 20µg/ml proteinase K in 50mM Tris, 5mM EDTA (freshly diluted from stock) at room temperature for 7.5 minutes. Slides were washed twice in PBS for 5 minutes and refixed in 4% PFA for 5 minutes, quickly rinsed in distilled water and placed in a glass trough containing 200 ml 0.1 M triethanolamine pH 8.0 and a stir bar. While stirring, 500µl of acetic anhydride was added to the dish and left stirring for 10 minutes. The sections were then washed for 5 mins each in PBS and saline, followed by dehydration through ethanol series and air drying. The slides were used the same day for hybridisation.

Hybridisation was carried out in the following buffer: 60% formamide, 0.3 M NaCl, 20 mM Tris pH 8.0, 5 mM EDTA, 10 mM $NaPO_4$ pH 8.0, 10% Dextran sulphate, 1 x Denhardt's, 0.3 mg/ml tRNA, 0.3 mg/ml rRNA, 0.3 mg/ml polyA.

Probes were taken up in this buffer at 3 ng/µl/Kb probe complexity. 3-10µl of hybridisation mixture was used to overlay each specimen which was then covered with a siliconised coverslip. Slides were placed horizontally in a plastic slide box containing a tissue paper soaked in 50% formamide, 5 x SSC and sealed with tape. The box was then placed in a dry incubator at 52^0C for 16-18 hours.

The next day, slides were washed for 15 minutes in 5 x SSC, 20 mM 2-mercaptoethanol at 50^0C to remove the coverslips and then put through the following washes:

1) 50% formamide, 2 x SSC, 0.2M 2-mercaptoethanol, 65^0C, 20 minutes.

2) RNase buffer (0.5 M NaCl, 10 mM Tris, 5 mM EDTA) 37^0C, 10 minutes, three times.

3) 20µg/ml RNase A in RNase buffer 37^0C, for 30 minutes

4) RNase buffer 37^0C, 15 minutes.

5) 50% formamide, 2 x SSC, 0.2M ß-mercaptoethanol, 65°C. 20 minutes.

6) 2 x SSC, 50°C, 15 minutes.

7) 0.1 x SSC, 50°C, 15 minutes.

The slides were then dehydrated through an ethanol series, air dried and dipped in 0.1% gelatine, 0.01% $CrK(SO_4)$, followed by air drying under dust free conditions.

2.2.8 Autoradiography

Ilford K5 nuclear track emulsion was diluted on the day of use by melting 6ml of emulsion shreds at 50°C with an equal volume of 2% glycerol in H_2O. Each experimental slide was dipped into emulsion, left to drain vertically for two seconds and dried horizontally in a large light tight box after the back of the slide has been wiped clean.

When the slides were dry (usually after about three hours), they were transferred to a light tight plastic slide box containing dessicant. Exposure at 4°C was usually for 3 days, one week or three weeks.

Before development, the slides were allowed to warm up to room temperature for two hours. Slides were developed for 2.5 minutes in

Ilford phenisol, development was stopped in 2% acetic acid (30 seconds), followed by a 30 second rinse in distilled H_2O and fixation in freshly made 30% sodium thiosulphate for five minutes. The slides were then washed under running tap water in the dark room for one hour, rinsed in distilled H_2O and stained with haematoxylin, destained as required and mounted in neutral mounting fluid.

2.2.9 Microphotography

Results were photographed on an Olympus BH2 microscope equipped with dark-field optics and a manual C35DA-2 35mm camera. Kodak Panatomic X film (ISO 32) was used and developed in Kodak D76 developer for 5 minutes at 20°C. Dark-field images were photographed by using a 6-8 fold underexposure and bright-field images were exposed at the normal setting, using a LBD-2N blue filter.

In later experiments double-exposure colour photography was used to demonstrate the distribution of silver grains. The Olympus IF550 filter was used on light field exposure with a 4-6 fold underexposure on dark-field exposure with the Cibachrome .40M Red filter.

CHAPTER 3 RESULTS

3.1 CLINICAL STUDY

3.1.1 Birth prevalence

286 patients affected with cleft lip, cleft palate or both were identified amongst the 187,321 total births to women residing in the West of Scotland between 1st January 1980 and 31st December 1984. The birth prevalence for this time period was thus estimated as 1.53 +/-0.09 per 1000 total births ($^{o}/_{oo}$TB). 51/286 (17.8%) of the cases had cleft lip (CL), 147/286 (51.4%) had cleft palate (CP) and 88/286 (30.8%) had cleft lip and palate (CLP). Table VI shows the total birth prevalence of each of these sub-groups. 268/286 (93.7%) of the patient were liveborn out of the 186,139 live births during the study period, thus the live birth prevalence can be calculated as 1.44 +/-0.09 per 1000 live births ($^{o}/_{oo}$LB) for all facial clefts. Of the live born patients 51/268 (19.0%) had CL, 142/268 (53.0%) had CP and 75/268 (28.0%) had CLP (Table VI).

TABLE VI Birth Prevalence of Facial Cleft Subgroups

Cleft Type	Total Cases	Birth Preval. $^{o}/_{oo}$TB+/-2SD	Liveborn Cases	Birth Preval. $^{o}/_{oo}$LB+/-2SD
CL	51	0.27 +/-0.038	51	0.27 +/-0.038
CLP	88	0.47 +/-0.050	75	0.40 +/-0.046
CP	147	0.79 +/-0.065	142	0.78 +/-0.064

In 168/286 (58.7%) of the patients the facial cleft represented an isolated congenital anomaly. If patients with isolated Pierre Robin sequence (PRS) are included the proportion rises to 187/286 (65.4%). However the birth prevalence of isolated facial clefts calculated by excluding all cases with associated major congenital malformtions (p_i) may be an

underestimate as it will ignore the possibility of randomly associated congenital anomalies. Table VII shows the corrected birth pevalence (p_c) of the sub-groups calculated using the method of Czeizel & Tusnady (1984) (see section 2.1.3).

TABLE VII Birth Prevalences of Isolated Facial Clefts

Cleft Type	No.isolated cases	p_i $^{o}/_{oo}$TB+/-2SD	p_c $^{o}/_{oo}$TB
CL	46	0.25 +/-0.036	0.26
CLP	57	0.30 +/-0.040	0.31
CP	65	0.35 +/-0.043	0.36
CP + PRS	84	0.45 +/-0.049	0.47

3.2 CLEFT LIP WITH OR WITHOUT CLEFT PALATE

3.2.1 Side of cleft

139 cases of cleft lip with or without cleft palate (CL(P)) were identified, 51/139 (36.7%) had cleft lip only (CL) and 88/139 (63.3%) had cleft lip and palate. Of these 139 patients with CL(P), 86 (61.9%) had unilateral defects, two cases had midline defects (1.4%) and in 41 cases (29.5%) the defect was bilateral. In 10 cases (7.2%) the nature of the lip defect was not recorded in the hospital or pathology records and no photographic or clinical follow up was available.

In the unilateral CL(P) cases (where laterality was recorded) left sided defects were more common accounting for 55/86 (63.9%) of this group. This excess was statistically significant ($x^2 = 6.7$ $p<0.01$). Table VIII shows that left sided lesions are commoner in the sub-groups with no associated abnormalities although only the isolated CL(P) male excess achieves statistical significance ($x^2=7.8$ $p<0.05$). In CL(P) cases with

associated abnormalities there was no difference in unilateral CL laterality and bilateral defects constituted a higher proportion of the defects with 20/43 (46.5%) with bilateral defects, 10/43 (23.3) with right sided and 13/43 (30.2%) left sided defects. This difference however was not statistically significant (YM x^2=3.54 p<0.1 p>0.05).

TABLE VIII Laterality of lip defect in the CL(P) group

Side	Isolated		Associated Abn	
	CL	*CLP*	*CL*	*CLP*
Right	14	10	1	6
Left	21	27	2	5
Bilateral	5	19	0	17

3.2.2 Severity of cleft

The distribution of defect severity, quantified using the method of Jensen et al. (1988), is presented graphically in Figure 7. 43/51 of the cleft lip only group could be scored, 32/43 (74.4%) had scores of 2 or 3. 84/88 of the cleft lip and palate group could be scored, 69/84 (82.1%) had a score of 4 compared to 7/43 (16.2%) of the cleft lip only group. This difference was highly statistically significant (YM x^2=48.64 p<0.001).

3.2.3 Sex differences

In the 139 CL(P) group there was a statistically significant excess of males accounting for 90/139 (64.7%) of the cases (YM x^2=8.36 p<0.01) thus giving a male:female ratio (m:f) of 1.84:1 (cf.1.052:1 for total births in this period). If cases with associated major anomalies are excluded the sex difference in the 103 remaining cases becomes more marked with males representing 74/103 (71.8%) with a m:f=2.55:1 (YM x^2=15.45

Figure 7

Severity Scores in the Facial Cleft Groups

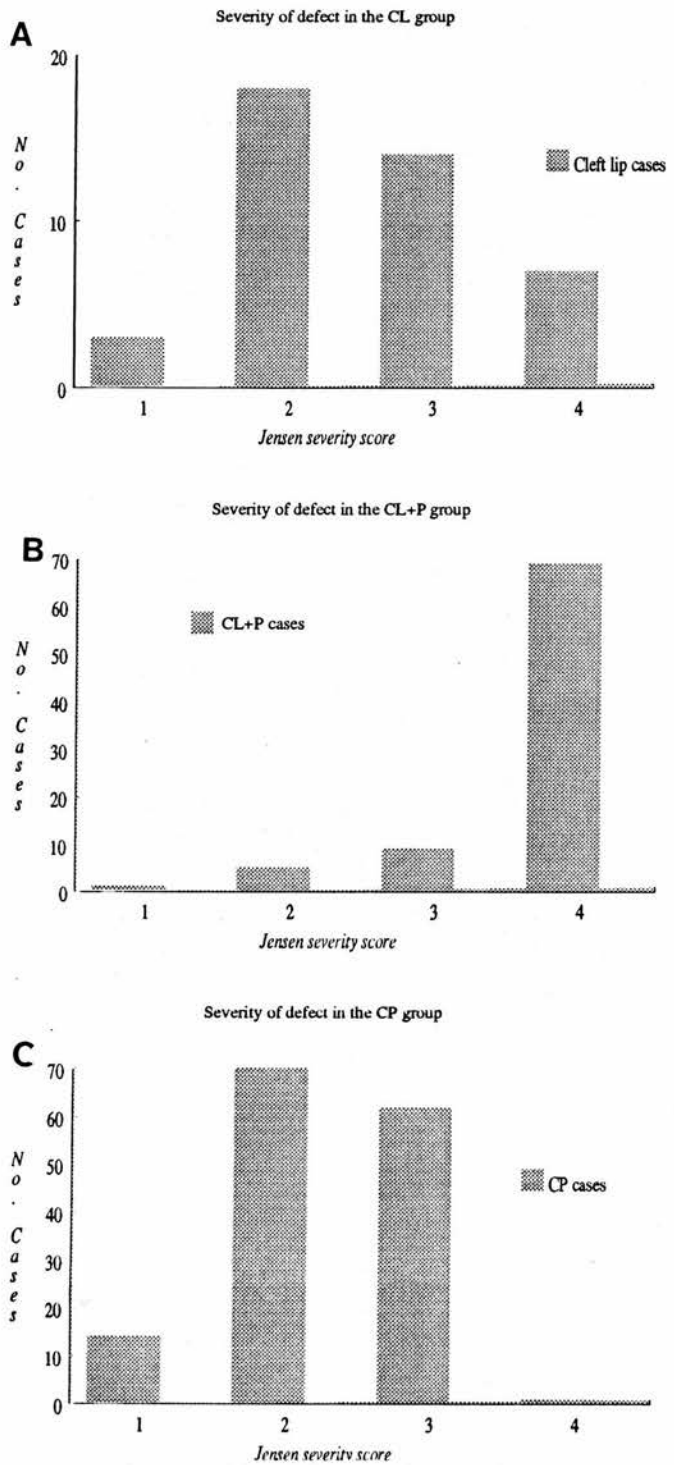


FIGURE 7. Severity score in the facial cleft groups. Jensen severity scores of the ascertained patients displayed graphically. (A) Cleft lip group (B) Cleft lip and palate group (C) Cleft palate group.

$p<0.002$). There is no statistically significant sex difference in the group with associated major abnormalities (Table IX).

TABLE IX Sex differences within the facial cleft group

Defect	Isolated		Associated	
	Male	*Female*	*Male*	*Female*
CL	35	11	3	2
CL+P	39	18	13	18
CL(P)Total	**74**	**29**	**16**	**20**
CP excl. PRS	30	40	27	27
PRS	5	14	4	0

3.2.4 Geographical distribution in CL(P) group

The geographical distribution assessed by the mother's Health Board of residence at time of birth is shown in Table X. None of the differences in birth prevalence between Health Boards achieve statistical significance.

3.2.5 Seasonal distribution of births in the CL(P) group

The temporal distribution by month of birth is shown in Figure 8. Using either the Edwards parametric test (Edwards, 1961) or non-parametric rank-sum (Hewitt et al. 1971) tests no significant cyclic trend was demonstrated within the group (or subgroups) when compared with figures for total births in the West of Scotland over the same time period.

Figure 8

Seasonality in the Facial Cleft Groups

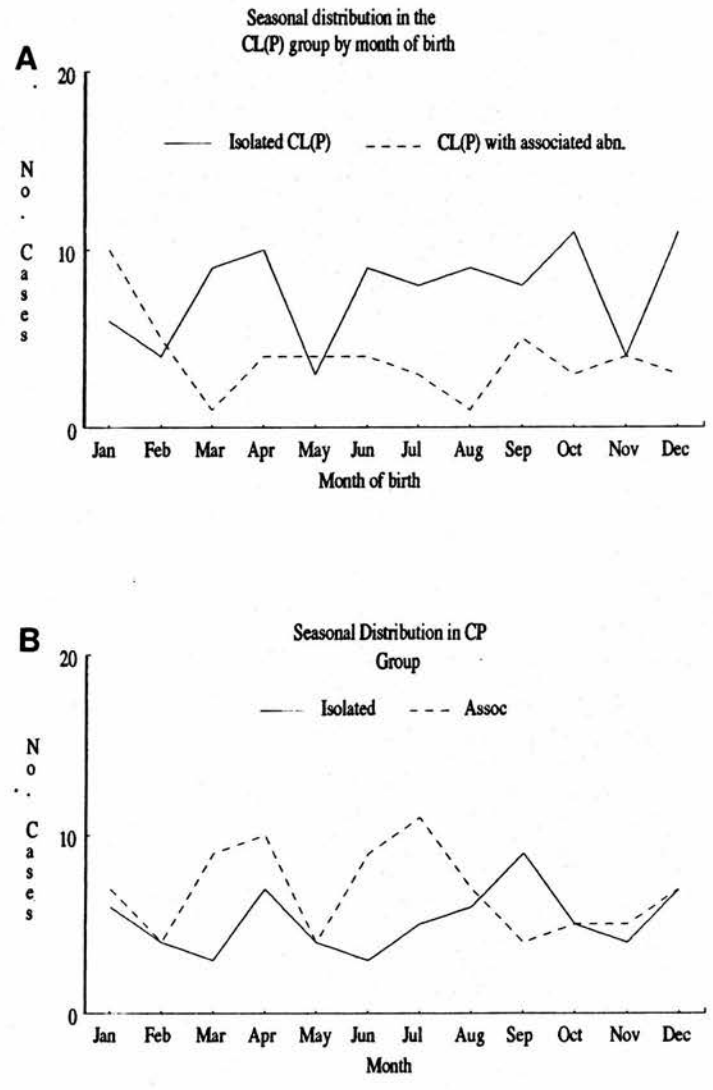


FIGURE 8. Seasonal variation in the facial cleft groups. Graphical representation of the births to women resident in the West of Scotland during the study period by month. (A) Cleft lip with or without cleft palate group (B) Cleft palate group

3.2.6 Outcome

13/139 cases in the CL(P) group were stillborn, all of the stillborn infants with CL(P) had major associated abnormalities (Appendix C). Of the 126 live born cases in the CL(P) group 10 had died by 1/1/90, 8 of the 10 fatalities had major associated anomalies (Appendix D). The proportions of stillbirths (YM x^2=2.89 p<0.1 p>0.05) and early deaths (YM x^2=0.08 p>>0.1) are not statistically significantly different from those found in the CP group (see (section 3.3.5).

TABLE X Health Board of residence at time of birth

Health Board	Total Births (1980-84)	CL(P) cases (1980-84)	CP cases (1980-84)
Argyll & Clyde	30518	24 [0.79]	26 [0.85]
Ayrshire & Arran	24899	22 [0.88]	19 [0.76]
Dumfries&Galloway	8787	9 [1.02]	6 [0.68]
Forth Valley	17340	13 [0.75]	8 [0.46]
Greater Glasgow	65257	45 [0.69]	59 [0.91]
Lanarksire	40520	26 [0.64]	29 [0.72]
Total	187321	139 [0.74]	147 [0.79]

[n] = Birth prevalence ($^{o}/_{oo}$TB)

3.2.7 Associated abnormalities

48/139 (34.5%) of the CL(P) group had associated abnormalities. 36/48 (75%) were major malformations. Single gene defects account for 8/139 (5.7%) chromosomal aberrations 5/139 (3.6%) and identifiable malformations 8/139 (5.7%). A further 17/139 (12.2%) had multiple malformations that could not be identified as part of a particular

syndrome. The known diagnoses in this group are summarised in Table XII. The abnormalities associated with the CL(P) in the unknown group are summarised in Table XI. Details of these patients can be found in Appendix E.

3.3 THE CLEFT PALATE GROUP

3.3.1 Severity of the palate defect

147 cases of cleft palate (CP) were identified in the study, in two of the cases the degree of cleft was not recorded. 130/145 (89.6%) had severity scores of 2 or 3, represented graphically in Figure 7.

TABLE XI Abnormalities associated with facial cleft cases with no identified syndrome

Defect	CL(P) Cases n=17 [%]	CP Cases n=22 [%]
Craniofacial defects	7 [41]	1 [5]
Congenital heart defects	5 [29]	9 [41]
Eye anomalies	5 [29]	6 [27]
Abdominal defects	4 [23]	4 [18]
CNS malformations	4 [23]	3 [14]
Skeletal defects	4 [23]	3 [14]
Mental retardation	3 [17]	4 [18]
Genitourinary anomalies	1 [5]	4 [18]
Pulmonary malformations	1 [5]	- [0]
Distal limb anomalies	1 [5]	- [0]

3.3.2 Sex distribution in the CP group

81/147 (55.1%) of cases in the CP group are female giving a male:female ratio of 0.81:1. This female excess is not statistically significant (YM x^2=3.11 p>0.05). There was however a statistically significant excess of females in the subgroup of CP without associated abnormalities (m:f=35:54=0.65:1)(YM x^2=6.19 p<0.05). In the subgroup of CP with associated abnormalities there was no female excess (m:f=31:27=1.15:1). The m:f ratios in the isolated CP group are highly statistically significantly different from those found in the isolated CL(P) group (YM x^s=19.27 p<0.001) (Table VII). No statistically significant difference is found between the CP and CL(P) groups with associated abnormalities (YM x^2=0.41 p>0.1).

3.3.3 Geographical distribution in CP group

The geographical distribution of the cleft palate group by mother's Health Board of residence at time of birth is shown in Table VIII. No significant differences are seen in the total birth prevalence figures although Forth Valley would appear to have fewer cases of CP than would be predicted (YM x^2=1.78 p>0.1).

3.3.4 Seasonal distribution of births in the CP group

The temporal distribution in the CP group shown by month of birth is graphically summarised in Figure 8. There are no statistically significant differences in the whole group or the sub-groups when compared with the total births for the same period.

3.3.5 Outcome in the CP group

5/147 (3.4%) cases in this group were stillborn, all had associated abnormalities; details of this group are given in Appendix C. Of the 142 live born children with cleft palate, 14 (9.7%) had died by 1/1/90 (Appendix D), all had major associated abnormalities. Neither of these

TABLE XII Known diagnoses in the CL(P) group

Single Gene Defects	Chromosomal Defects	Malformation Syndromes
Myotonic dystrophy	Trisomy 13 (3)	Schisis association (3)
Van der Woude (2)	Unbalanced reciprocal translocation	Holoprosencephaly
X-linked hydrocephalus	De novo reciprocal X,10 translocation	Goldenhars (2)
Hay Wells syndrome		Di Georges syndrome
Hypertelorism-Mictrotia-Clefting syndrome		Dandy Walker syndrome
Popliteal web syndrome		
Griegs syndrome		

TABLE XIII Known diagnoses in the CP group

Single Gene Defects	Chromosomal Disorder	Malformation Syndromes
Diastrophic dystrophy	De novo 9p+	Pierre Robin anomalad (23)
Velo-cardio-facial syndrome	De novo t(X,9)	Schisis association (4)
Campomelic syndrome	De novo 11q-	VATER association
Kneist dysplasia	De novo t(3,5)	1st/2nd arch syndrome (2)
Ectrodactyly-Ectodermal dysplasia-Clefting syndrome	Trisomy 18 (2)	
Van der Woude syndrome	Trisomy 18 mosaic	
Sticklers syndrome (2)	De novo complex rearrangement	
Treacher Collins syndrome		
Oro-Facio-Digital syndrome I		
Crouzon syndrome		
Myotonic dystrophy		

figures are significantly different from those found in the CL(P) group (see section 3.2.6).

3.3.6 Associated abnormalities

86/147 (58.5%) of the CP group had associated abnormalities, 58/86 (67.4%) had major malformations associated with cleft palate. Single gene defects accounted for 12/147 (8.2%), chromosomal abberations 8/147 (5.4%), malformation syndromes 30/147 (20.4%) and teratogenic agents 1/147 (0.7%, fetal alcohol syndrome). The known diagnoses are summarised in Table XIII. A further 22/149 (14.8%) had associated malformations that could not be placed in a particular syndrome diagnosis, the anomalies occurring in these 'unknown' malformation syndromes are shown in Table XI, no significant differences are seen in the associated abnormalities occuring in the CL(P) group and those seen in the CP group. Details of the patients with unknown syndromes can be found in Appendix E.

3.4 RNA LOCALISATION STUDIES

The spatial and temporal distributions of RNAs encoding the three related growth factors, TGFß1, ß2 and ß3 were investigated during murine palatogenesis from 11.5 to 15.5 days gestational age. *In situ* hybridisation was performed on 5-7 µm coronal sections of the murine embryonic head using radioactive gene-specific probes complementary to each transcript (see 2.2.6). As a negative control, a human TGFß1 sense probe was employed which gave no specific hybridisation signal (Fig. 9B). In total 50 embryos from 12 litters were studied (Table XIV).

No specific hybridisation of any probe was seen in the very early palatal shelves at 12.5 days gestational age (Fig.9C-H). The first appearance of TGFß transcripts occurred at the late vertical shelf stage. In this study two patterns of RNA distribution were seen at this time and later in shelf development. TGFß1 and ß3 are expressed in the medial edge epithelia,

whereas TGFß2 RNA is localised in the underlying mesenchyme.

3.4.1 Late vertical palatal shelves.

At 13.5 days gestation the TGFß1-specific probe showed strong hybridisation to the submandibular gland (Fig.10C). At this stage, the palatal shelves, which are growing vertically, show no specific hybridisation to this probe.

The first TGFß gene to show high level expression in the palatal processes is that for TGFß3 (Fig.10G,H). This gene is expressed in the epithelial component of the palatal shelves in the region that will give rise to the future medial edge epithelium. In several embryos examined, the spatial extent of the hybridisation signal is identical. TGFß3 expression is more extensive in the vertical epithelium facing the tongue and stops abruptly on the oral side. In the most anterior region of the oro-nasal cavity, the epithelium of the nasal septum, which is also destined to fuse with the palatal shelves, shows high level TGFß3 expression (Fig.10G).

There is a low level of hybridisation of the TGFß3 probe to the mesenchyme of the tongue, mandible and the upper regions of the maxillary processes. The mesenchyme immediately adjacent to the medial edge epithelium is, however, markedly devoid of autoradiographic signal. This observation was also made at later stages (see Fig.11,12).

At this early stage, TGFß2 does not appear to be specifically expressed in the palatal processes, although a characteristic signal is seen in the differentiating olfactory epithelium of the nasal process (Fig.10E,F). On some sections, small regions of the medial edge epithelia show hyperplasia which represent the developing palatal rugae. The epithelial cells within these small blebs of stratified epithelium (Fig.10E) express very high levels of TGFß2 RNA (Fig.10F), whereas there is no hybridisation with either TGFß1 or ß3.

Figure 9

TGF beta ISOFORMS IN EARLY PALATOGENESIS

Localisation of TGF beta1, _2 and _3 in early palatal shelves. Coronal sections are presented from 12.5-day embryos through the anterior third of the palatal shelves. A,C,E and G are bright-field images, B,D,F and H are dark-field. (A) Bright-field image showing tongue (to),palatal shelves (ps) and nasal septum (ns). (B) Dark-field images showing non-specific hybridisation with sense probe. (C) Bright-field image of D (D) TGF beta1 probe showing no specific hybridisation (E) Bright-field image showing TGF beta2 hybridisation to the nasal epithelia (ne) (F) TGF beta2 showing no specific hybridisation in the palatal shelves (G) Bright-field of H (H) Non-specific mesenchmal hybridisation of the TGF beta3 probe.

Figure 9

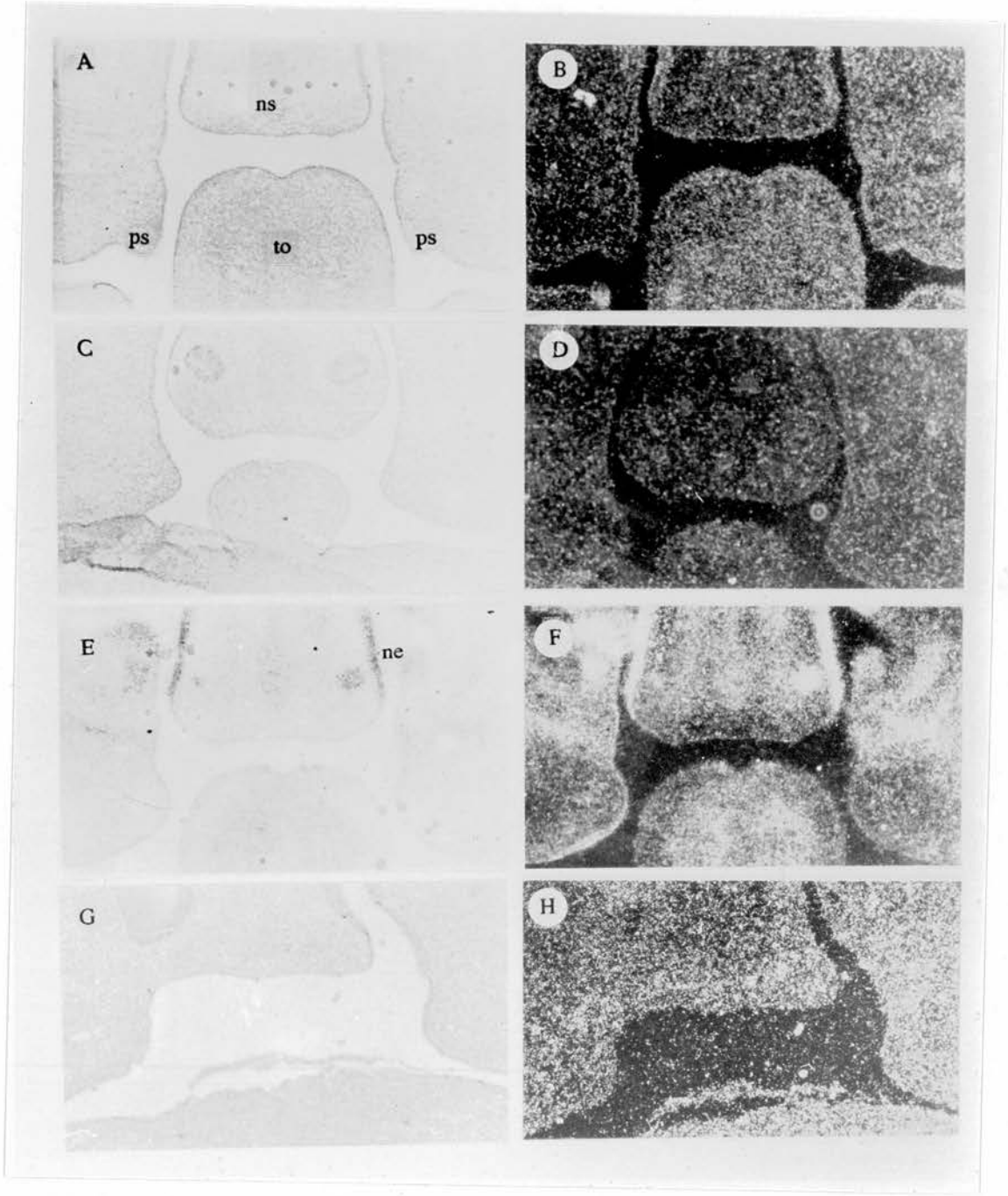
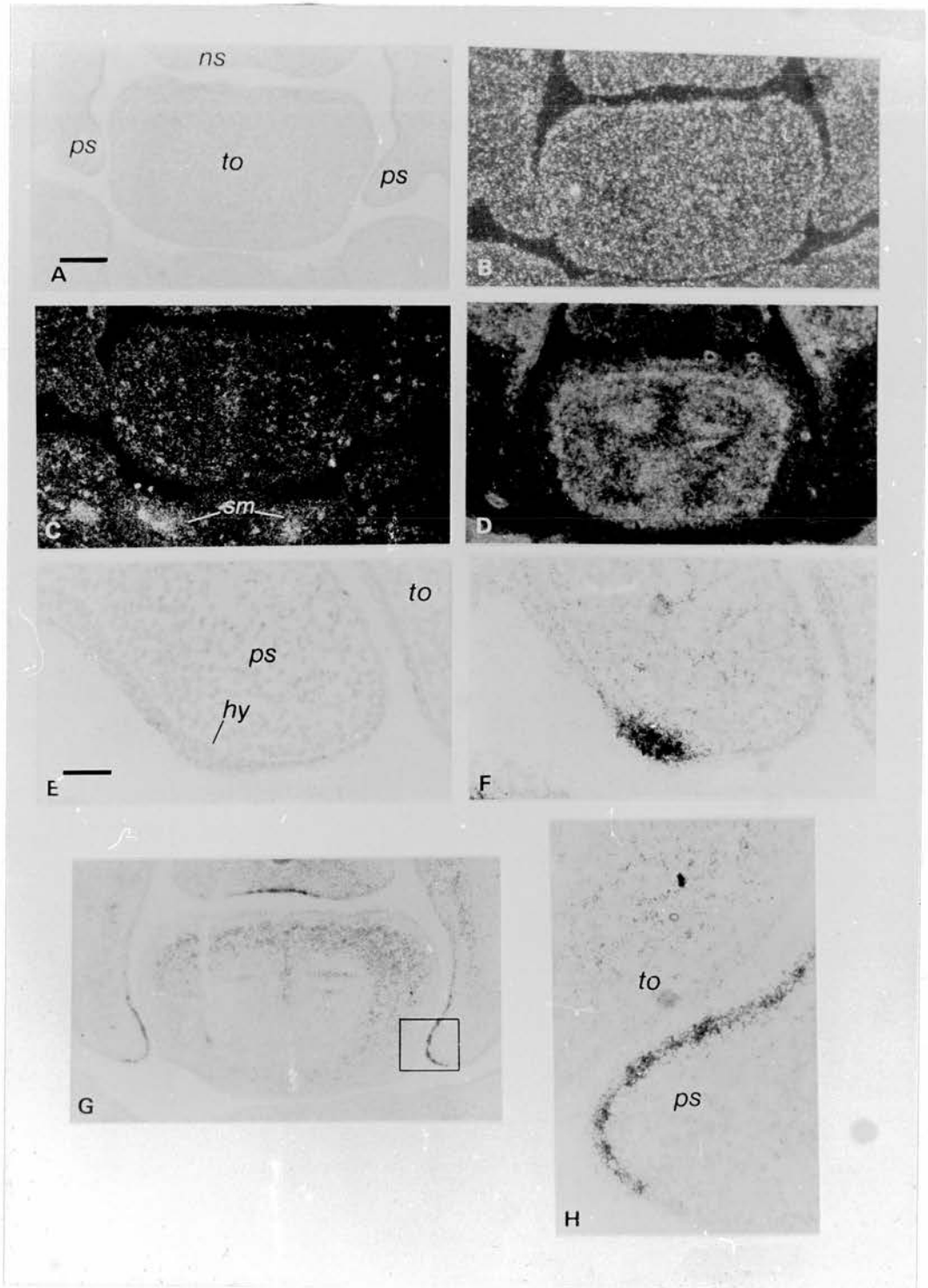


Figure 10

TGF beta ISOFORMS IN VERTICLE PALATAL SHELVES

Coronal sections are presented from 13.5-day embryos through the anterior third of the vertical palatal shelves. A and E,F,G,H are bright-field images, B,C,D are dark-field. (A) Bright-field image showing tongue (to),palatal shelves (ps) and nasal septum (ns). (B) Dark-field images showing non-specific hybridisation with sense probe. (C) TGF betal probe showing hybridisation to the submandibular gland (sm). (D) Non-specific mesenchymal hybridisation of TGF beta2 probe with localised area of expression in the epithelium of the left palatal shelf. (E) High-power image of adjacent section to D showing localised hyperplasia (hy) in the epithelia of one palatal shelf (ps). (F) High-power view of epithelial expression in D. (G) Hybridisation of the TGF beta3 probe to the medial epithelia of the palatal shelves and the oral epithelium of the nasal septum. (H) High-power view of the palatal shelf (ps) epithelial expression (boxed area) in G. Scale bar (A,B,C,D,G) represents 50 micrometers, scale bar (E,F,H) 200 micrometers.

Figure 10



3.4.2 The horizontal palatal shelf.

After elevation of the palatal shelves at around 14.0 days gestation, the medial edge epithelia come into almost immediate contact (Fig.11A). At this stage, TGFß1 is now detectable in the medial edge epithelia in the same cells that express TGFß3 (Fig.11B). TGFß1 expression is also seen in regions of membramous ossification is also seen within the developing maxilla (Fig.11B).

By this time the quantity of TGFß3 RNA in the medial edge epithelium has increased considerably. There is striking hybridisation with this probe in both in the medial edge epithelia and the epithelium of the oral aspect of the prefusion anterior nasal septum (Fig.11F,G,H).

TGFß2 RNA is excluded from the palatal epithelium, but a gradient of expression is seen within the mesenchyme beneath the medial epithelium, with highest expression levels immediately adjacent to the epithelium (Fig.11E).

3.4.3 The fusing palate.

With fusion of the medial edge epithelia to form the epithelial seam, the expression patterns established in the horizontal shelves continue. TGFß1 (Fig.12B) and TGFß3 (Fig.12D,G,H) RNAs are localised to the epithelial cells of the seam. This expression is lost as the seam disrupts and the cells lose their epithelial phenotype by transformation into mesenchymal cells.

TGFß2 transcript levels increase and are are now easily seen in the mesenchyme on either side of the seam (Fig.12C,E,F). The RNA distribution is asymmetric with a higher concentration towards the nasal side of the palate.

Figure 11

TGF beta ISOFORMS IN HORIZONTAL PALATAL SHELVES

All sections are from 14.0-day embryos. (A) Coronal bright-field image of horizontal, middle third palatal shelve (ps) medial edge epithelia (me) with the nasal septum (ns) above and oral cavity (oc) below. (B) Adjacent section to A with TGF beta1 probe hybridising to the maxilla (ma) and medial edge epithelia (me). (C) Bright-field paramedial sagittal section through posterior medial edge epithelia (me). (D) Dark-field image of C with TGF beta1 probe specifically hybridising to the medial edge epithelium. (E) Dark-field image of coronal section TGF beta2 hybridising to the medial and nasal palatal mesenchyme. (F) TGF beta3 hybridising to the medial edge epithelia on adjacent section to A. (G) Dark-field image of coronal section through middle third of palate, posterior to F, TGF beta3 probe hybridises to the medial edge epithelia. (H) High-power view of the palatal shelves (ps) and medial edge epithelia (me) expression in G (boxed area). Scale bar (A,B,C,D,E,F,G) represents 50um and in (H) 200um.

Figure 11

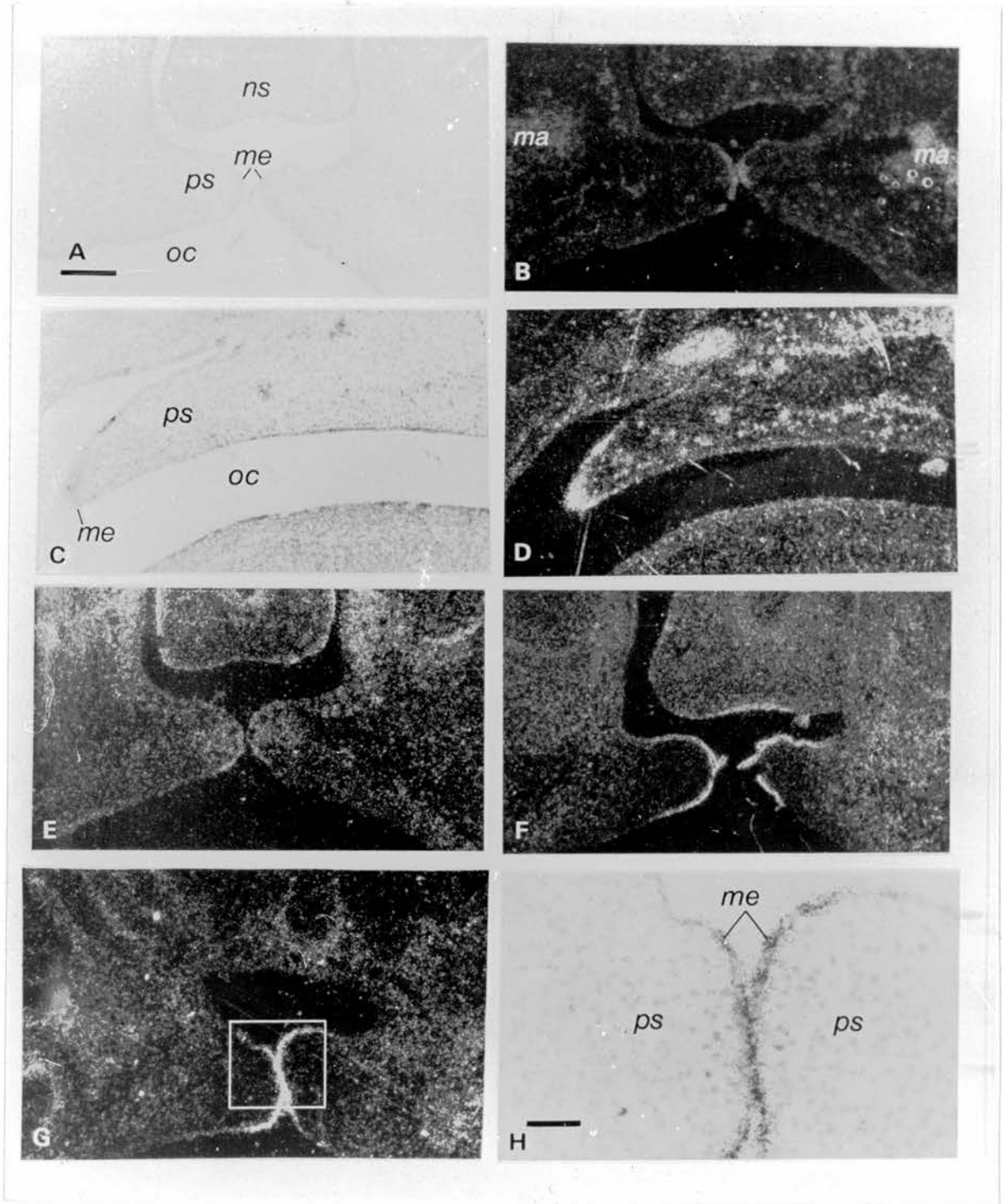
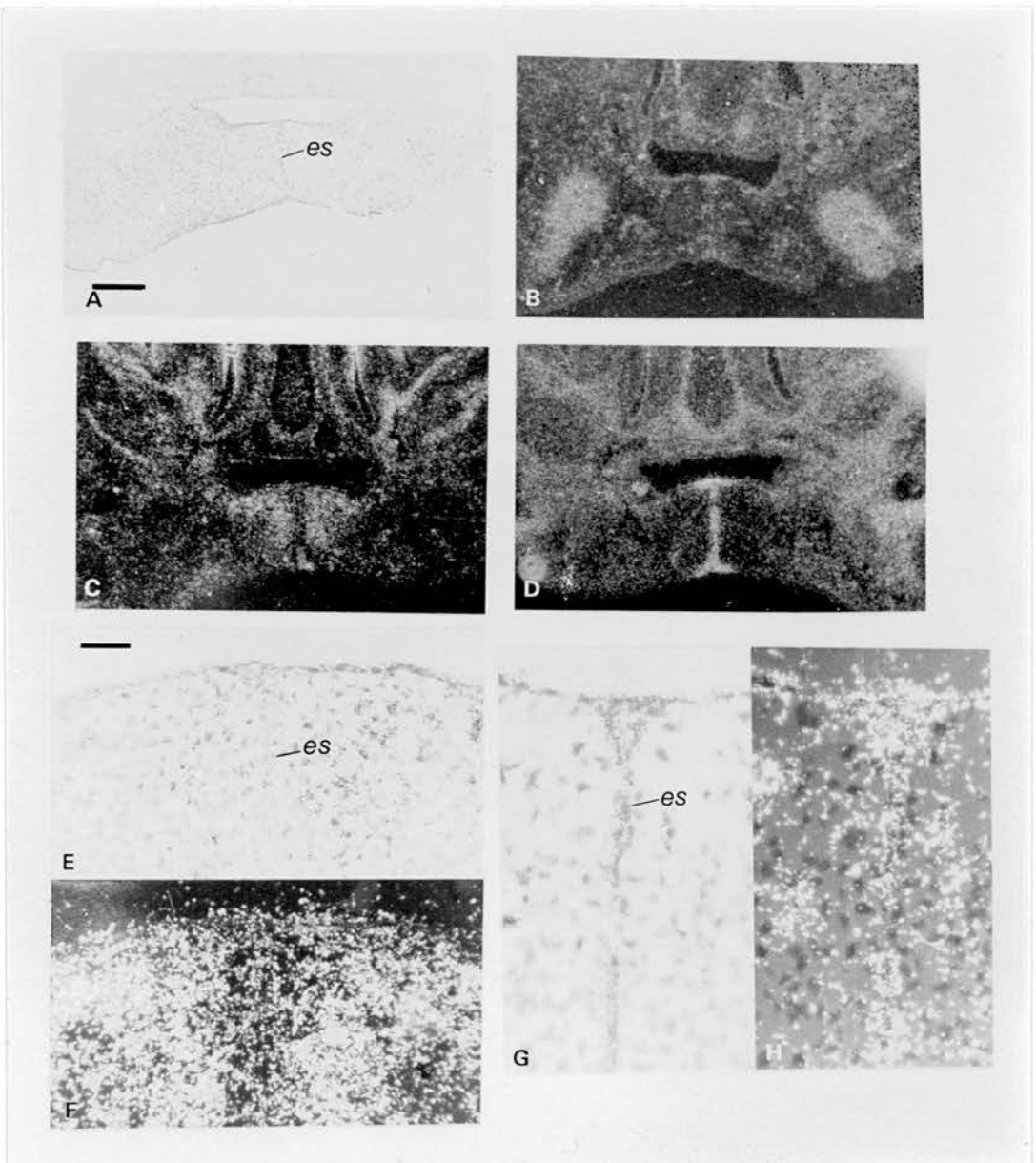


Figure 12

TGF beta ISOFORMS IN THE FUSING PALATE.

All sections are coronal through the middle third of the fusing palate of 14.5-day embryos. (A) Bright-field image of fusing palate with the midline epithelial seam (es). (B) Dark-field image showing TGF beta1 faint hybridisation to the epithelial seam. (C) Dark-field image of TGF beta2 hybridisation to the medial and nasal palatal mesenchyme. (D) Dark-field image of specific hybridisaton of TGF beta3 to the epithelial seam. (E) High-power bright field image of the epithelial seam (es) in C showing absence of TGF beta beta2 expression in the seam. (F) Dark-field image of E. (G) High-power image of the epithelial seam (es), showing hybridisation only where the seam is intact. (H) Dark-field image of G. Scale bar (A,B,C,D) represents 50um and in (E,F,G,H) 200um.

Figure 12



3.4.4 The maturing palate.

After fusion, TGFß2 continues to be expressed in a diffuse region of the mesenchyme around the midline of the palate (Fig.13C). Postfusion, TGFß3 expression also switches to become primarily mesenchymal (Fig. 13D). Hybridisation is seen in the perichondrium of the nasal septum as well as in the mesenchyme of the anterior secondary palate (Fig.13D). TGFß1 expression in the maturing palate is limited to areas of ossification within the palate and around the nasal processes (Fig.13B).

3.5 Retinoic acid-treated embryos

In this part of the study sections of mouse embryos treated with RA were hybridised with anti-sense radiolabelled riboprobes to detect TGFß1, ß2, ß3 RNA. Two groups of treated embryos were used in this study. The first group, named the Glasgow embryos (Fig.14A-H), were generated from C57BL/6 mice and treated by the author at GD 12 with 100mg/kg of RA and collected on GD 14. A total of 30 embryos from 6 litters were studied (Table XIV). The second group, named the Bethesda sections (Fig.15, 16 & 17) were supplied by Dr. Barbara Abbott (NIH, Maryland) as part of a collaborative study. These were generated from C57BL/6 crosses with the embryos treated with 100mg/Kg of retinoic acid on GD 10 (see section 2.2) and collected on either GD 12.5 or GD 14.5. A total of 20 embryos from 6 litters were studied (Table XIV).

3.5.2 The Glasgow Sections

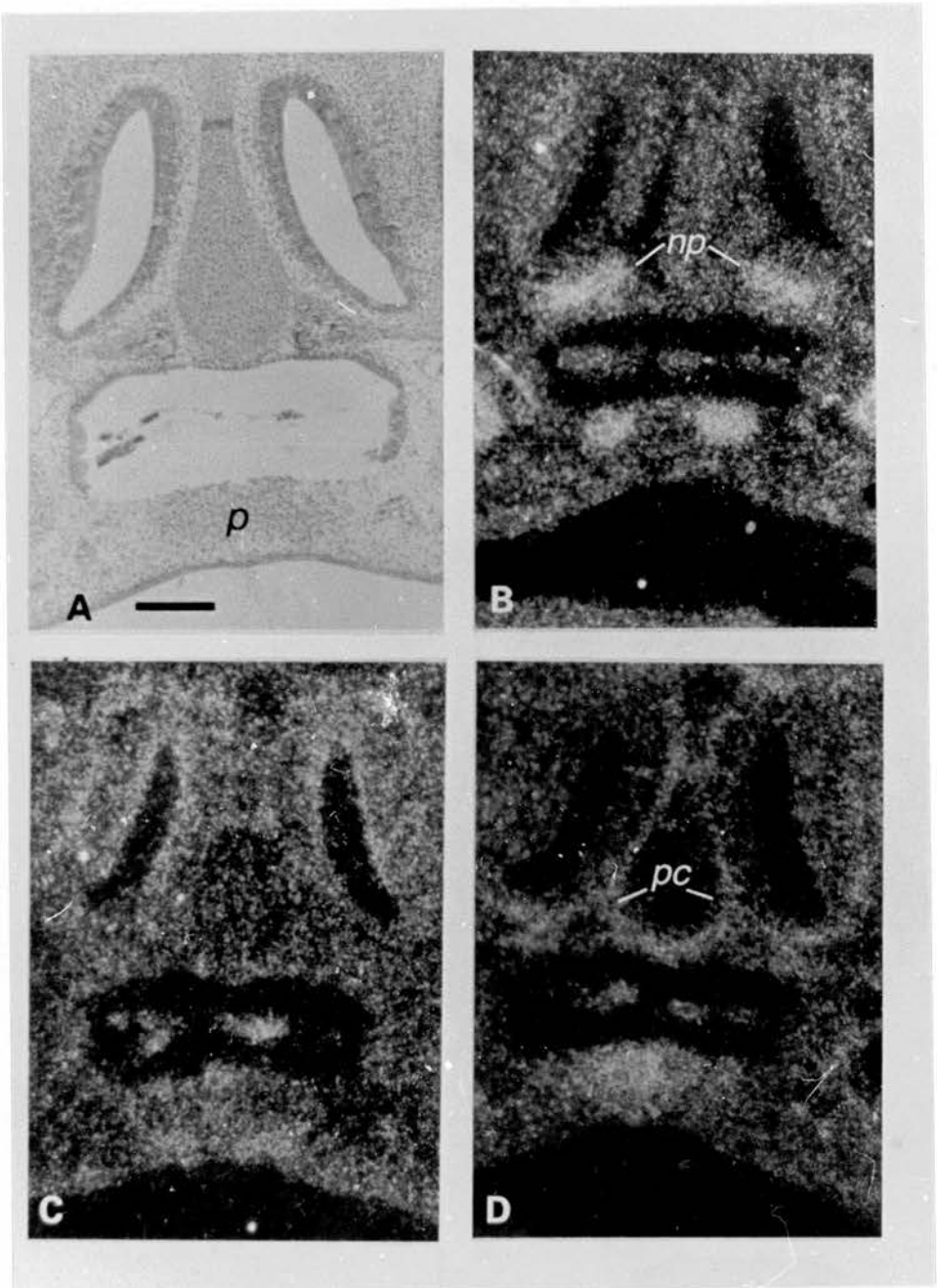
The control (untreated) embryos collected on GD 14 from this group showed no difference in developmental stage or hybridisation pattern to those reported in section 3.4, with TGFß1 (Fig.14C) and TGFß3 (Fig. 14E) being expressed in the MES and TGFß2 in the medial edge palatal mesenchyme. The RA-treated embryos the palatal shelves do not appear hypoplastic, however, they are phenotypically abnormal as the palatal shelves have not undergone reorientation and are therefore still vertical in position. In spite of this developmental delay the hybridisation of TGFß3

Figure 13

TGF betas IN THE MATURING PALATE

Sections have been cut coronally through the middle third of the palates of 15.5-day embryos. A is a bright field image and B,C,D are dark-field. (A) The maturing palate (p). (B) TGF beta1 probe hybridising to the areas of ossification within the palate and around the nasal processes (np). (C) Diffuse hybridisation of the TGF beta2 probe to the mesenchyme around the midline of the palate. (D) Specific perichondrial (pc) hybridisation of the TGF beta3 probe around the nasal septum and the midline mesenchyma of the palate. Scale bar represents 50 um.

Figure 13



to the MEE is clearly seen (Fig.14H), with no specific TGFß2 RNA localisation (Fig.14F). TGFß1 probe hybridises to the submandibular gland but not to the MEE (Fig.14D).

3.5.3 The Bethesda Sections

The control (untreated) embryos from this group of slides showed no difference in expression pattern from the NIH/Parkes crosses (see section 3.4) either on GD 12.5 (Fig.15A,C,E) or GD 14.5 (Fig. 16A,C,E). In the treated embryos collected on GD 12.5, the palatal shelves were considerably less distinct than those in the control embryos (Fig.15B,D,F). There was no specific pattern with any probe at this gestation with either control or RA treated sections (Fig.15A-F).

In the sections from the RA treated embryos collected on GD 14.5, the palatal shelves are noticably hypoplastic with grossly abnormal orofacial structure (Fig.16B,D,F). These changes are similar to those previously reported by Abbott et al. (1989). Although the treated sections are phenotypically abnormal the hybridisation signal for all three TGFßs follows the 'normal' patterns established in the NIH/Parkes (section 3.4) and untreated C57BL embryos (see above). TGFß3 RNA is abundant in the MEE (Fig. 16F), with TGFß2 hybridising to the medial edge palatal mesenchyme (Fig.16D) and a TGFß1 low-level signal is seen in the same palatal areas as TGFß3 (Fig.16B). The control embryos at this gestation have normal, elevated shelves and have formed midline epithelial seams (Fig.16A,C,E).

One difference in hybridisation pattern between the control and RA-treated embryos is the localisation of TGFß2 RNA to the MEE as well as the medial edge palatal mesenchyme (Fig.17A). TGFß2 is also seen in the developing rugae of the RA-treated palatal shelf (Fig.17B).

Figure 14

GLASGOW RA-TREATED EMBRYOS

Coronal sections through the anterior third of the palate of embryos harvested on 14.5 GD. Double exposure images are shown with red representing the silver grains. Untreated C57BL embryo (A) and RA-treated embryo (B) showing no specific hybridisation of the sense probe. (C) Untreated embryo showing hybridisation to the developing maxilla (ma) with slight signal in the MES of the palatal shelves (ps) with the TGF beta1 probe (D) RA-treated showing hybridisation of the TGF beta1 probe to the submandibular gland only (E) Untreated embryo probed with TGF beta2 showing slight hybridisation to the medial edge palatal mesenchyme (F) RA-treated showing non-specific mesenchymal hybridisation of the TGF beta2 probe to the mesenchyme surrounding the submandibular gland (G) Untreated embryo probed with TGF beta3 with strong hybridisation to the medial epithelial seam (mes) (H) RA-treated embryo with hybridisation of TGF beta3 probe to the MEE.

Figure 14

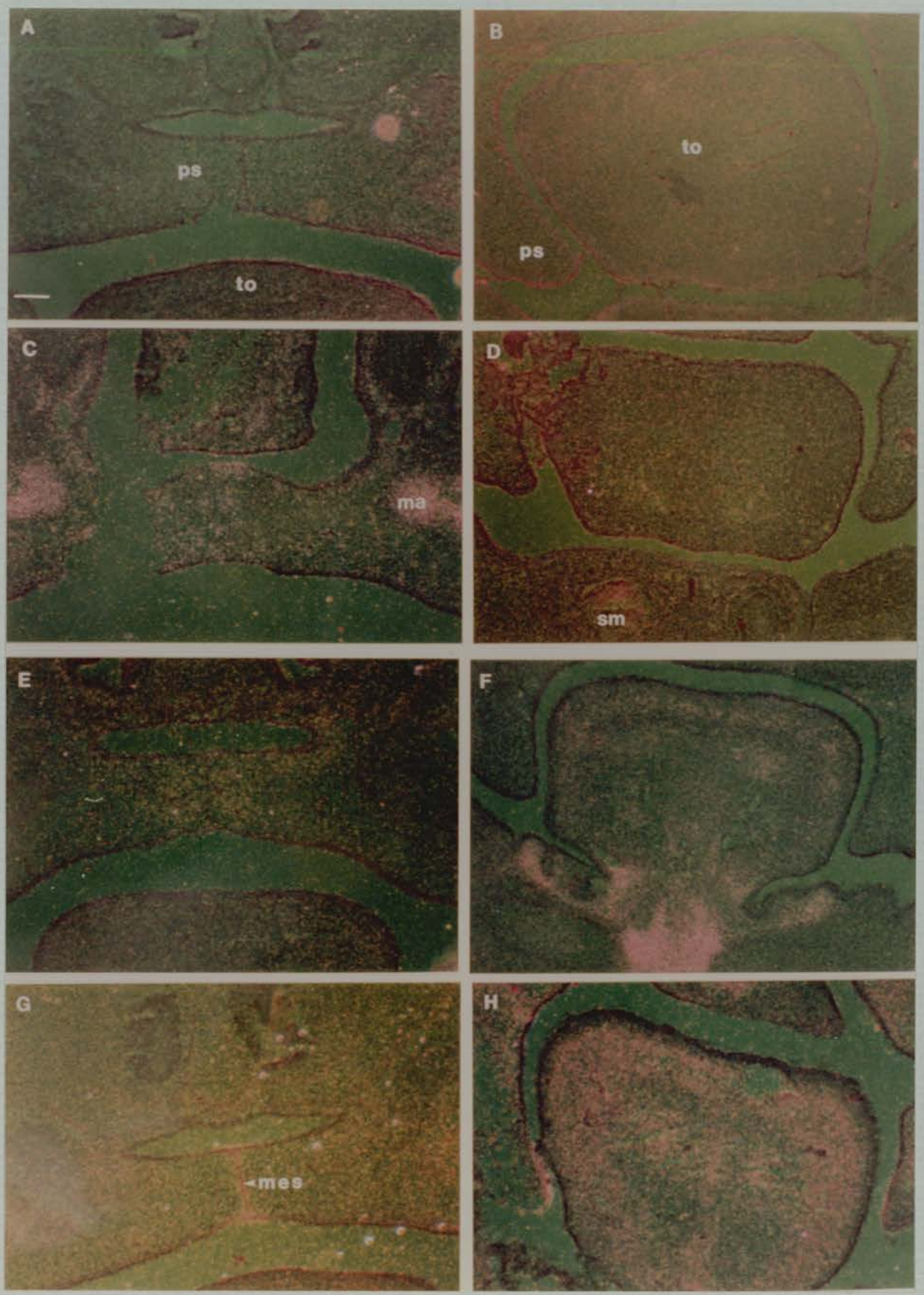


Table XIV Numbers of embryos studied

Study	*No. of Litters*	*No. of embryos*
NIH/Parkes study	12	50
Glasgow *C57B controls*	3	15
C57B RA-treated	3	15
Bethesda *C57B controls*	3	10
C57B RA-treated	3	10

In summary a distinctive pattern of the three mammalian TGFß isoform expression has been demonstrated. This expression pattern is not perturbed, in a major way, by retinoic acid-treatment which would lead to cleft palate.

Figure 15

BETHESDA RA-TREATED EMBRYOS 12.5 GD

Coronal sections through the anterior third of the palate of embryos harvested on 12.5 GD. Double exposure images are shown with red representing the silver grains. Untreated C57BL embryo (A) and RA-treated embryo (B) showing no specific hybridisation signal in the palatal shelves (ps) with the TGF beta1 probe. (C) Untreated embryo probed with TGF beta2 showing hybridisation to the mesenchyme and epithelia of the nasal septum (D) RA-treated showing hybridisation of TGF beta2 probe to chondrogenic blastema (ch) of the snout (E) Untreated embryo probed with TGF beta3 with no specific RNA localisation (F) RA-treated embryo with hybridisation of TGF beta3 probe to the maturing chondrogenic blastema.

Sections B,D,F in this figure appear to be anterior to sections A,C,E this effect is due to the remarkable reduction in snout length in the RA-treated embryos.

Figure 15

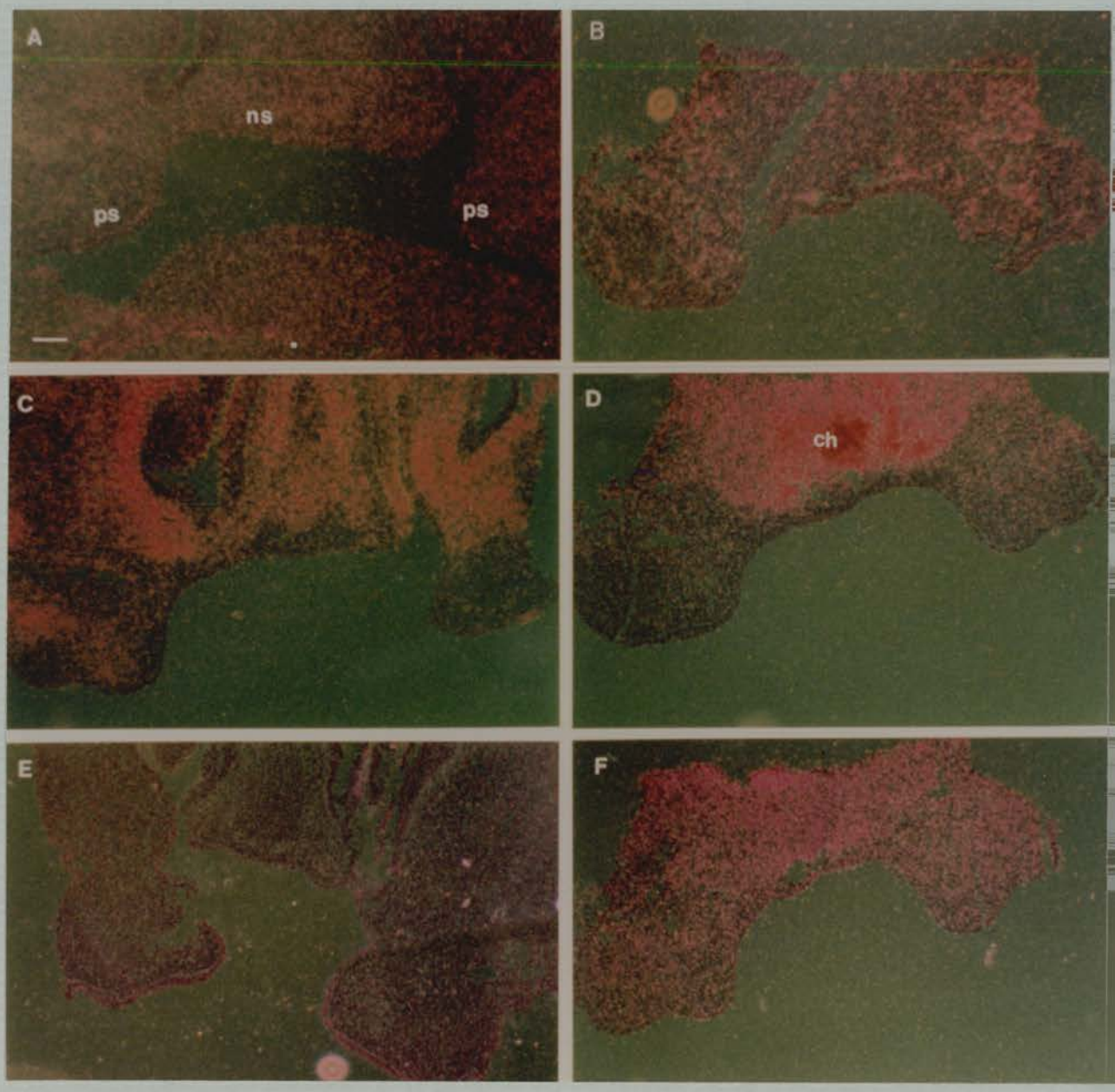


Figure 16

BETHESDA RA-TREATED EMBRYOS 14.5 GD

Coronal sections through the anterior third of the palate of embryos harvested on 14.5 GD. Double exposure images are shown with red representing the silver grains. Untreated C57BL embryo (A) and RA-treated embryo (B) showing hybridisation to the developing maxilla (ma) with slight signal in the MEE of the palatal shelves (ps) with the TGF betal probe. (C) Untreated embryo probed with TGF beta2 showing hybridisation to the medial edge palatal mesenchyme and epithelia of the nasal septum (D) RA-treated showing hybridisation of the TGF beta2 probe to the MEPM, the MEE and the rugae (r) of the palatal shelves (E) Untreated embryo probed with TGF beta3 with strong hybridisation to the medial epithelial seam (mes) (F) RA-treated embryo with hybridisation of TGF beta3 probe to the MEE.

Figure 16

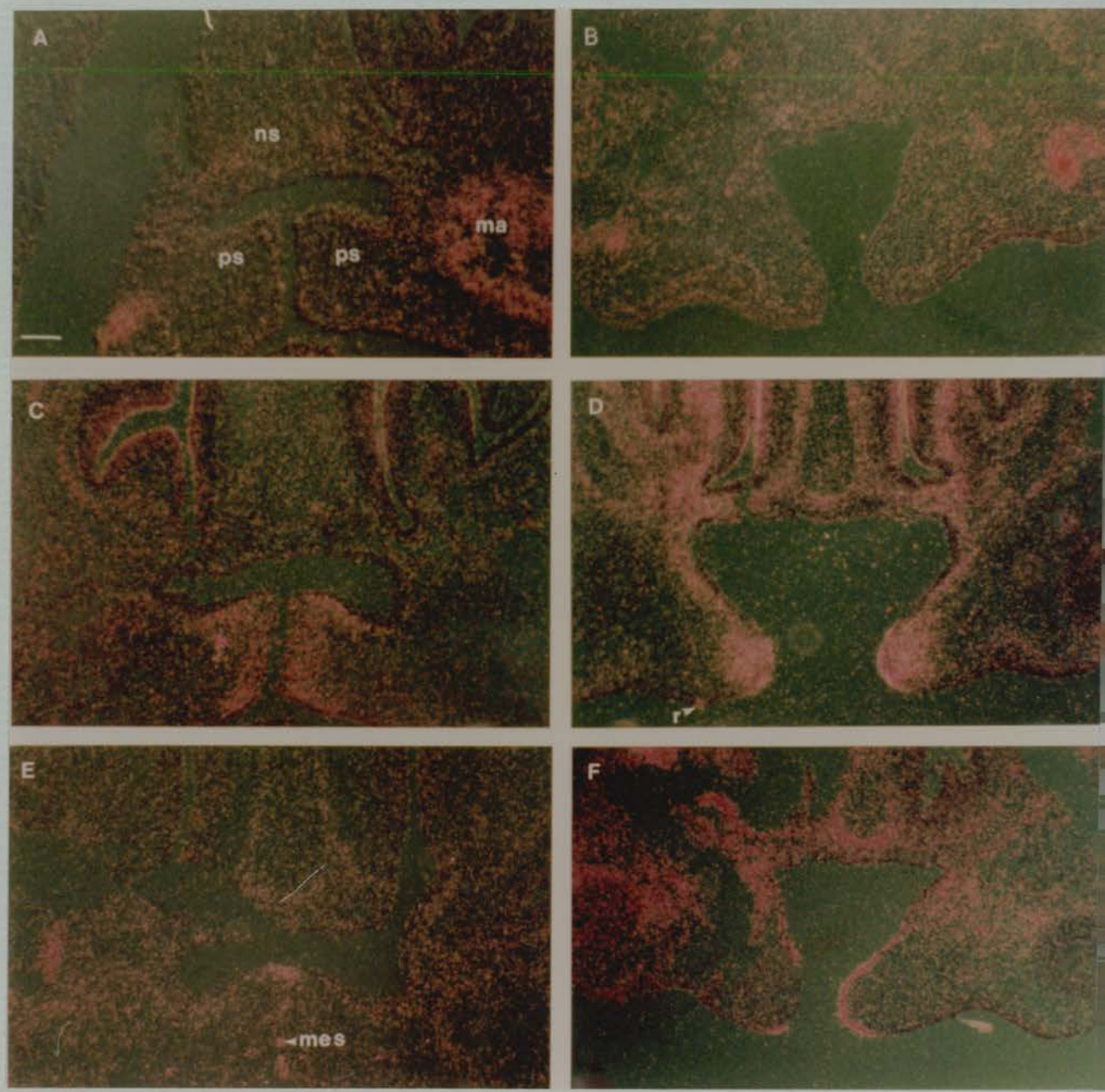
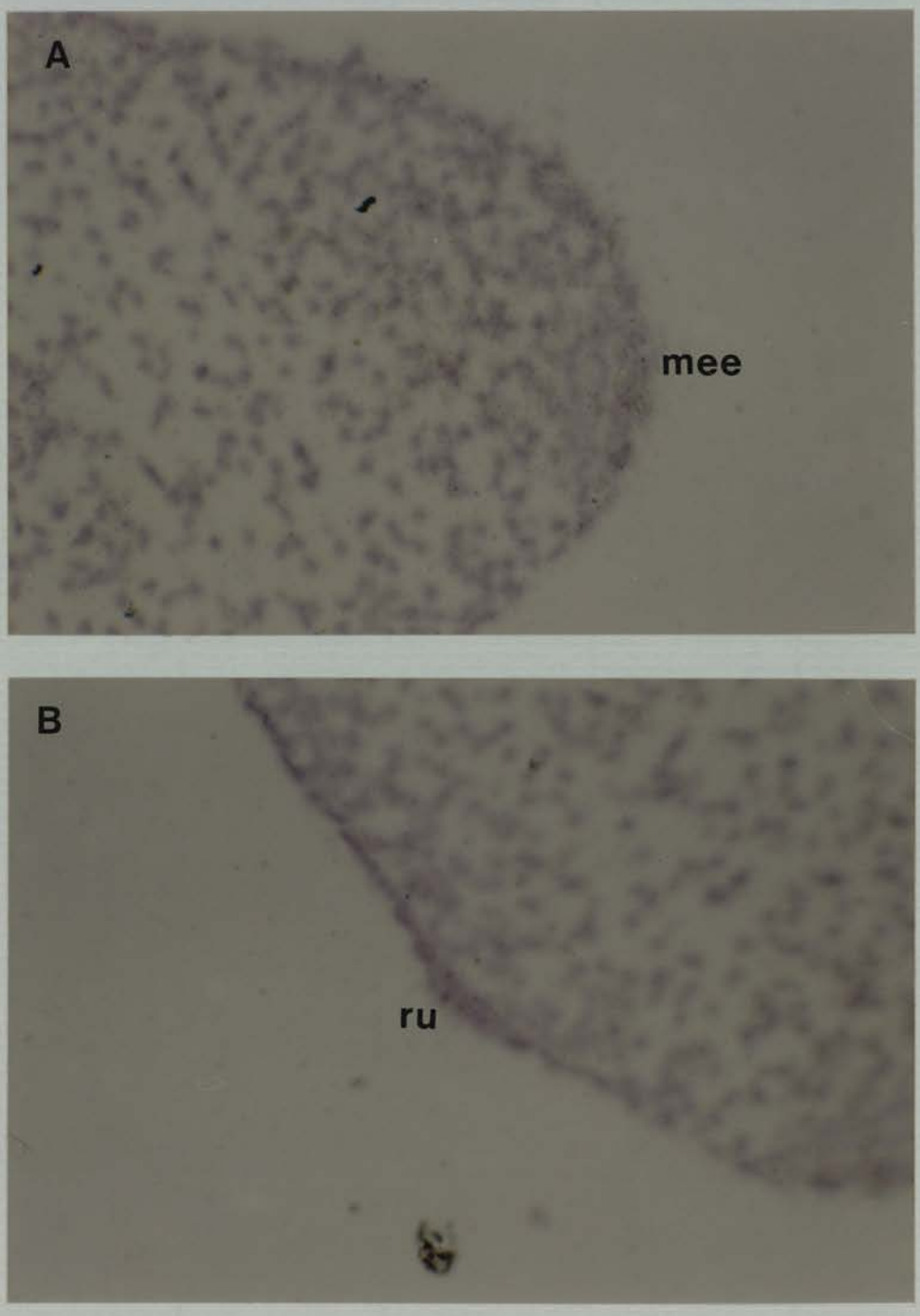


Figure 17

TGF beta2 IN RA-TREATED EMBRYOS

High-power, light-field image of the palatal shelve in Fig.16D. (A) TGF beta2 hybridisation to the mesenchyme of the palatal shelf and the medial edge epithelium (mee). (B) TGF beta2 hybridising to the developing palatal rugae (ru).

Figure 17



CHAPTER 4 DISCUSSION

4.1 THE CLINICAL STUDIES

As one of the most common easily recognisable birth defects in humans, it is perhaps surprising that it took until 1942 for a large scale, well ascertained study of the clinical and family aspects of facial clefts to appear (Fogh-Anderson 1942). Although earlier studies of facial cleft incidence had been published (Davis, 1924), the Danish study was the first to recognise cleft lip with or without cleft palate (CL(P)) and isolated cleft palate (CP) as distinct entities and to provide unbiased data on recurrence risks. Since that time the developmental aspects and etiology of these defects have been the subject of many hundreds of scientific and clinical studies. In spite of this explosion of interest it would appear that these studies have posed many more questions than they have answered.

4.1.1 Birth prevalence

Since Fogh-Anderson published his landmark study there have been more than one hundred studies reporting the birth prevalence of facial clefts in many different populations. Fourteen of the most widely quoted of these are summarised along with the results from the present study in Table XV. Some of these studies use either hospital based records (Fogh-Anderson, 1942; Bear 1976; Bonaiti *et al.* 1982) or birth certificates (Woolfe *et al.* 1963; Tenconi *et al.* 1988) as ascertainment data. The limitations of single-source data have already been discussed and are essentially those of under-reporting and ascertainment bias (section 1.4.2). As these problems will hamper any population based study, the use of multiple sources of information has become accepted as the optimal method of data collection and many recent studies attempting to calculate true birth prevalence, including the present one, have used diverse sources of ascertainment (Beckman & Myrberg 1972; Welch & Hunter 1980; Wormsley & Stone 1987; Jensen *et al.* 1988).

Before comparing these data an attempt to estimate the efficiency of

TABLE XV Reported birth prevalences of facial clefts

Author	Year	Total Incidence (No. Cases)		CL	CL+P	CP	Population
Present study	1990	1.53	(286)	0.27	0.47	0.79	West of Scotland
Fogh-Anderson	1942	1.5	(625)	0.375	0.75	0.375	Danish
Neel	1958	2.68	(171)	--	--	--	Japanese
Longnecker *et al.*	1965	0.59	(163)	--	--	--	Black Americans
Stevenson *et al.*	1966	1.21	(528)	0.98		0.23	WHO Multicentre Study
Chung & Myrianthopoulus	1968	1.82	(24)	1.34		0.48	European Americans
		0.82	(64)	0.41		0.41	Black Americans
Lowry & Renwick	1969	3.17	(64)	0.25	2.45	0.44	Candian Indians
		1.63	(737)	0.38	0.71	0.54	European Canadians
Beckman & Myberg	1972	1.72	(298)	1.17		0.55	Sweden
Saxen & Lahti	1974	1.72	(599)	0.83		0.86	Finland
Czeizel	1980	1.43	(1437)	0.37	0.71	0.35	Hungary
Welsh & Hunter	1980	2.00	(507)	--	--	--	Canada
Bonaiti *et al.*	1982	1.52	(646)	0.96		0.56	French
Womersley & Stone	1987	1.56	(247)	0.21	0.54	0.81	West of Scotland
Jensen *et al.*	1988	1.89	(678)	0.64	0.74	0.51	Danish
Tenconi *et al.*	1988	--	(203)	0.92		--	Italy
Naguib *et al.*	1989	1.56	(166)	0.63	0.78	0.15	Kuwait

Total Incidence: This is given as the number of cases per 1000 total births.

(No. Cases): This figure refers to the total number of cases used in the study.

ascertainment and identify any ascertainment bias in the present study was made. Figures for facial cleft birth prevalence estimated from the Glasgow EUROCAT registry were compared with those from the non-Glasgow areas. The Glasgow EUROCAT registry is one of seventeen EEC-funded registries established throughout Western Europe. It catalogues all congenital malformations born to mothers resident in the Greater Glasgow Health Board (GGHB) using multiple sources of ascertainment and it is subjected to systematic evaluation of the accuracy and completeness of its data (Stone, 1979). The register has access to many privileged sources of information and considerable manpower and is thought to have approaching 100% ascertainment for facial clefts (Wormesly & Stone 1987). Thus, in what can be considered the well ascertained group of GGHB residents, the birth prevalence of facial clefts during the study period was 1.61 per 1000 total births ($^{0}/_{00}$TB) and 1.51 per 1000 live births ($^{o}/_{oo}$LB), whereas in non-Glasgow residents they were 1.48 $^{o}/_{oo}$TB and 1.40 $^{o}/_{oo}$LB. Although these proportions are not statistically significantly different (YM x^2=0.37 p>0.1), they suggest an under-ascertainment in the order of 8% in non-GGHB areas.

No one source for this possible under-ascertainment in the non-GGHB areas could be identified as no pattern difference between the various subgroups was found (e.g. CL(P):CP ratio, stillbirth rate, %age with associated abnormalities). It is interesting that Czeizel & Tusnady (1984) have noted very similar differences in estimates of the facial cleft birth prevalence in the Hungarian population between the figures collected in the city of Budapest where the study was based and a greater number of investigators were working to complete ascertainment, and figures collected in the areas outside Budapest. They too failed to detect any particular subgroup that was under-ascertained. In summary, although there is some suggestion of under-ascertainment in the present study, there would appear to be no ascertainment bias.

From the published reports of facial cleft birth prevalence summarised in Table XV it can be seen that the total birth prevalence varies widely from 0.59 to 3.17 per 1000 total births. There would appear to be consistent

racial differences in birth prevalence of CL(P), being lower than Caucasians in African races (0.49 - 0.82 per 1000 total births) and considerably higher than Caucasians in Oriental races (eg. Japanese, North American Indians) at around 3 per 1000 total births (see section 1.4.1). Within populations of European origin, however, there is considerably less variation in the reported total birth prevalence of facial clefting, which range from 1.42 +/- 0.04 to 1.89 +/- 0.07 per 1000 total births. These differences are relatively small and could be caused by low sample size in individual studies, a difference in the efficiency of ascertainment or a genuine genetic or environmental difference between the populations. The first of these would seem an unlikely explaination as there is no overlap at the 95% confidence intervals between the two examples quoted above. A difference in efficiency of ascertainment between studies, although possible, would also seem unlikely as both the higher (Jensen *et al.* 1988) and the lower of these birth prevalence figures (Czeizel, 1980) were conducted by highly respected investigators using diverse sources of information to maximise ascertainment in well ordered highly cooperative communities.

The third possible explanation for the differences within European populations in overall birth prevalence is that there is genuine environmental and/or genetic heterogeneity that alters population thresholds for facial clefting. This view may be supported by the significant differences in the CL(P):CP ratio between European populations. These differences can be exemplified by comparing the CL(P):CP ratios Scottish and Finnish studies with those in other groups. In the present study, the CL(P):CP ratio was 0.94:1, this is similar to the ratios obtained in two previous Scottish studies of 0.92:1 (Womersley & Stone 1987) and 1.2:1 (Drillien *et al.* 1966) and the ratio of 0.96:1 reported from Finland (Saxen & Lahti 1974). The Scottish and Finnish figures do, however, differ significantly from all other reported European figures with an average ratio of 2.3:1 (range 1.71:1 - 3.09:1)(x^2=23.45 $p<0.01$). It would seem unlikely that these figures represent an ascertainment bias, as this would be likely to cause an under-ascertainment of less obvious cleft palate as compared to cleft lip

with or without cleft palate. It is probable that this is a genuine but, as yet, inexplicable difference between these groups. Beckman & Myrberg (1972) reported a total incidence of 1.82 per 1000 total births in the Swedish population, this total birth prevalence is similar in Northern, Central and Southern counties of Sweden . The CL(P):CP ratio, however, varied being 1.46:1 in Northern counties, 1.82:1 in Central counties and 3.72:1 in Southern counties. They cited possible ethnic heterogeneity due to an admixture of Lapps and Finns as a possible explanation, however, it is equally possible that environmental factors may be responsible. It would appear, therefore, that there is a relative excess of isolated cleft palate in the more Northern regions of Europe.

4.1.2 Severity scoring

Once a patient with facial clefting has been ascertained an accurate classification of the primary defect is desirable. If complex segregation analyses (Lalouel *et al.* 1983) are to be performed and/or empiric family recurrence data (Carter *et al.* 1982) calculated from this group, a method of scoring severity of the cleft is of particlular importance. Furthermore, some authors have suggested that knowledge of the distribution of cleft severity within the various subgroups may contribute to our understanding of the malformation aetiology (Jensen *et* al. 1988). Several different systems have been proposed to enable quantification of defect severity (Fogh-Andersen, 1942; Kernahan & Stark 1958; Harkins *et al.* 1962; Jensen *et al.* 1988). In the CL(P) group, however, the simplest method to estimate the distribution of severity is to calculate the ratio of cleft lip only (CL) to cleft lip and palate (CL+P), in the present study this ratio was 1:1.73 which is similar to ratios previously reported in European populations which range from 1:1.4 - 1:2.0 (for review see Czeizel & Tusnady 1984). However, a quantitative severity score allows more detailed analyses to be performed. In the present study, therefore, the method described by Jensen *et al.* (1988) as it was simple and reproducible (Jensen *et al.* 1988) and has the advantage that patients can be easily scored from clinical photographs.

The Jensen defect severity scores within both the CL and CP groups appeared to be normally distributed with the majority of patients in both these groups scoring either 2 or 3. However, the CL+P group showed a marked skew towards the more severe defects (score of 4). These are remarkably similar finding to those in the Jensen *et al.* study (1988). This distribution of severity of defect in the CLP group is in keeping with the proposal, based on embryological and recurrence risk data, that CL+P is a more severe manifestaion of CL. Czeizel & Tusnady (1984) have used the higher incidence of CL+P compared with CL in stillborn cases with facial clefts as further evidence that CL+P is the result of a more severe developmental insult than CL. Results from the present study agree with this assessment. Of the 13/139 (9.3%) of the CL(P) cases that were stillborn, all had CLP.

4.1.3 Sex differences

One of the many puzzling features of facial cleft epidemiology is the different sex ratios that have been reported between the two main groups of facial clefts. In the CL(P) group, the present study has shown a significant excess of affected males with a male:female ratio of 1.84 (YM $x^2=$ $p<0.01$). All European studies shown in Table XV have a similar excess of males with an average m:f ratio of 1.95:1 (range 1.82:1 - 2.11:1). However Japanese studies with a high birth prevalence of CL(P) have shown that either the male excess is markedly reduced (Tanaka *et* al. 1967) or that there is a slight excess of females in this group (Fujino *et* al. 1967). Analysis of the data on both CL(P) has ruled out a significant role for an X-linked gene (Csik & Mather, 1938; Chung *et al.* 1986). Thus, the explanation that is currently most widely accepted is a modification of the multifactorial or quasicontinuous model of inheritance (see section 1.6.2). In this model the cumulative effect of numerous, weak, independent factors, both genetic and environmental, have to exceed a threshold, which is different in male and female embryos, to cause a facial cleft (Carter 1965). Although this model has been supported by reports showing higher risks of recurrence in the relatives of affected females (Woolf 1971; Carter *et al.* 1982) there has

been no embryological basis suggested for the sex-modified threshold in CL(P).

Another possible explanation for the sex ratio heterogeneity within the CL(P) group has been raised with the introduction of segregation analysis under a unified model (Lalouel *et al.* 1983). Chung *et al.* (1986) reanalysed extensively researched family data from Denmark and Japan and found evidence for the action of a major autosomal recessive gene in the Danish populations that could not be determined in the Japanese data. Since a male excess is found in the Danish but is less apparent in the Japanese data, it is tempting to speculate that this, as yet, unidentified major gene acting in European populations is sex-limited, with a single threshold multifactorial model operating in the Japanese population, however no evidence supporting this theory is available at present.

The sex ratio within the CP group is markedly different. In the present study the m:f = 0.81:1 which is significantly different from that in the CL(P) group (YM x^2=10.7 $p<0.002$) but not from the total birth sex ratio (YM x^2=3.11 $p<0.1$ $p>0.05$). This slight excess of females has been a constant feature of reported studies in Table XV, with an average m:f ratio of 0.8:1 and a range of 0.5:1 - 1:1 (for review see Gorlin *et al.* 1976). Unlike the sex ratio in the CL(P) group this female excess would appear to be present in both Caucasian and Japanese populations but may be absent from African races (Shapiro *et al.* 1976). Like CL(P), a sex-modified multifactorial threshold for the sex differences in the CP group has been invoked (Carter, 1965). In support of this Burdi & Silvey (1969) has suggested a possible embryological basis for the threshold difference by reporting that the palate closes approximately one week later in female than male human embryos and suggesting that this may have an as yet undefined role in the aetiology of cleft palate. Watanabe & Endo (1989), however, found that although female mouse embryos had retarded development of palate and digital structures at 12 days gestation (period of early palatal shelf growth), there was remarkable catch-up of these developmental processes by 14.4 days gestation (time of shelf fusion) when female embryos were developmentally in advance of their

larger male equivalents. If this catch-up period exists in human embryos it may alter the cleft palate threshold for female embryos.

4.1.4 Laterality

Another intriguing aspect of this group of patients is the developmental lateralisation that is implied by the excess of left-sided defects in the unilateral cleft lip group. In the present study, 55/86 (64%) of the unilateral CP(P) cases had left sided defects. This is a highly statistically significant excess (x^2=6.7 $p<0.01$). All the reported studies that have analysed the defects by sidedness have given similar results (for review see Fraser, 1970). However no convincing explanations for this phenomenon have been advanced. The remarkable laterality of defect within CL(P) patients has led to interest in other systems that display laterality, thus leading to several studies of the concordance of handedness with side of defect in this group. Unfortunately, the four major studies of handedness in cleft lip patients (Tisserand, 1944; Rintala, 1985; Fraser & Rex 1985; Yorita & Melnick 1988) give conflicting results. Tisserand (1944) was the first study of this type and showed an excess of non-right-handedness (NRH) in patients with right-sided lip defects. Both Rintala (1985) and Yorita & Melnick (1988), however, found an excess of NRH in patients with left sided defects. Fraser & Rex (1985) failed to find any association between handedness and side of cleft. It is now obvious that large multicentre studies are required to clarify this situation. The supporters of the relationship between cleft lip side and contralateral handedness (Rintala 1985; Yorita & Melnick 1988) have suggested that any embryological insult affecting the cranial neural crest cells (that will go on to form the facial prominences) could also involve adjacent cells in the neural folds (that will go on to form the forebrain) and thus influence handedness. Although this is an interesting possibility it must remain speculative until the association between handedness and cleft side is proven or suggestive defects in forebrain development can be demonstrated. It is obvious that the above theory could not explain all laterality in facial clefts. Even if the association of cleft side and handedness is statistically proven it was

not present in the majority of patients in any of the three 'positive' studies.

4.1.5 Pre- and Postnatal selection

Evidence of early lethality in facial cleft groups is available from several studies (Iizuka, 1972; Bonaiti *et al.* 1982; Czeizel & Tusnady, 1984) and has been the source of some debate. A prenatal selective mechanism has been postulated as acting in this group (Czeizel & Tusnady 1984) and the high rate of stillbirths in the CL(P) (13/139 (9.3%)) and CP (5/147 (3.4%)) group in the present study would support this postulate. Further evidence for the existence of prenatal selection in facial clefts comes from the work of Iizuka (1973) who has shown an incidence of 1.56% of facial clefting (1.32% CL(P), 0.32% CP) in early human embryos collected from social terminations of pregnancy. Furthermore Bethmann & Eifert (1969) have shown a higher incidence of premature births among a group of patients with facial clefting which may be further evidence for prenatal selection. Niswander *et al.* (1972) have, therefore, proposed that an increased liability to embryonic death may be associated with a liability to facial cleft and have called this phenomenon 'developmental noise' (see below).

However Bear (1978) has pointed out that, if the above hypothesis was correct, then an increased embryonic loss would be seen in families with two or more affected individuals. On the contrary Bear (1978) has shown that the number of self-reported spontaneous abortions is greater in mothers of a single affected child than in mothers of two or more affected sibs. It has also been observed that in Caucasian populations, the normal male excess in individuals affected with CL(P) (see below) becomes less apparent in sibships with two or more affected members (Niwander *et* al.1972). Bear (1978) has, therefore, suggested that the equal sex ratio of affected members within multiply-affected sibships is due entirely to female embryos being spared, which is obviously at variance with what is predicted by the theory of developmental noise. The sex ratio, however, suggested that factors influencing embryonic

survival are sex-specific

It would appear from the relatively high mortality rate in both CL(P) (10/126 (7.9%)) and CP (14/142(9.7%)) groups in the present study, that post-natal selection may also may also be operating in the facial cleft groups, mainly in the first year of life. The deaths in the present study were all related to associated severe congenital anomalies and the mortality rates within the CL(P) and CP groups are similar to those previously reported by Mackeprang & Hay (1972).

4.1.6 Aetiology

The complete ascertainment of facial cleft patients within a community provides the investigator with an opportunity to study the various contributions of genetic and environmental factors in this highly heterogeneous group. Genetic factors can be assessed initially by looking for conditions with single gene or chromosomal aetiology (see below). The study of environmental factors is often more difficult as, in the absence of a specific teratogenic syndrome, few clues are available to the nature of the teratogenic factors. Seasonal variation with respect to congenital malformations has been useful as an indicator of an environmental component in the etiology and has the advantage that accurate dates of birth are almost always available in a patient data set (Edwards 1961). In the present study no cyclic trend could be detected in total group or the various subgroups using either the parametric test described by Edwards (1961) or the non-parametric method of Hewitt *et* al. (1971). Previous reports of seasonal variations in facial clefts have shown no significant cyclic trend in the CP group (Lutz & Moor 1955; Edwards 1961; Fraser & Calnan 1961), but have given conflicting answers in the CL(P) group. Lutz and Moor (1955) were the first to study seasonality in the CL(P) group reporting an excess of births between June and August. Edwards (1961) in a study from Birmingham and Fujino *et al.* (1963) in Japanese patients have however shown a significant excess of CL(P) patients born in early spring with a peak in March. Other studies have shown no significant seasonal differences

(Fraser & Calnan 1961; Drillien *et al.* 1966). The interpretation of these conflicting data is difficult, although the fact that two of the studies (Edwards 1961; Fraser & Calnan 1961) took place in the same country, during the same time period, but gave conflicing reports, must cast doubt on this phenomenon as a significant factor in facial cleft aetiology.

Many authors who have endeavoured to establish the causes of facial clefts have stressed the importance of cataloguing abnormalities and identifying syndromes associated with these defects (Fraser & Calnan 1961; Fraser 1970; Gorlin, 1976; Pashayan, 1983; Shprintzen *et al.* 1985). The identification of cases with unifactorial aetiology (single gene defects, chromosomal anomalies and teratogenic agents) is necessary, not only for accurate counselling of the family, but also to provide unbiased data for research use such as segregation analysis. Even if the aetiology of a malformation syndrome is not known, the diagnosis may provide valuable prognostic information. The published reports of the incidence of abnormalities associated with facial clefts are summarised in Table XVI.

As can be seen from Table XVI the percentages of facial cleft patients with associated abnormalities would appear to vary from 3% (Fraser, 1970) to over 50% (Shprintzen *et al.* 1985). It is not, however, appropriate to compare these figures directly. Fraser (1970) used the 3% figure as an estimate of the proportion of facial cleft patients that have identifiable syndromes rather than the total number with multiple congenital anomalies. In 1970 Fraser estimated there to be around 50 recognised syndromes associated with facial clefting, in 1990 there would appear to be around 230 syndromes (excluding chromosomal anomalies) with facial clefting as a recognised feature (Appendix A&B). Thus, it may be assumed that many more of the patients seen in 1970 may be recognised today as syndromic. Both Rollnick & Pruzansky (1981) and Shprintzen *et al.* (1985) took a different approach by studying all abnormalities associated with facial cleft, whether as part of recognised syndromes or not. This method has the advantage of giving an overall picture of associated abnormalities, but may lead to confusion

TABLE XVI Abnormalities associated with facial clefts

Study	Year	Cleft type	Associated anomalies (%age)	Single Gene Defects (%age)	Chromo-some anomalies (%age)	Teratogenic agents (%age)	Recognised malformation syndromes (%age)
Present study	1990	CL(P)	34.5	5.7	3.6	-	5.7
		CP	57.7	8.1	5.4	0.7	20.1
Shprintzen et al.	1985	CL(P)	46	13	3	3	5
		CP	59	20	1	5	9
Rollnick & Pruzansky	1981	CL(P)	35	-	-	-	22.7
		CP	54	-	-	-	33.4
Tenconi et al.	1988	CL(P)	22	3.0	8.9	-	3.4
Czeizel	1980	CL(P)	22.8	-	-	-	-
Fraser & Calnan	1961	CL	1.1	-	-	-	-
		CL+P	1.9	-	-	-	-
		CP	13.3	-	-	-	-
Knox & Braithwaite	1963	CL	2.8	-	-	-	-
		CL+P	7.3	-	-	-	-
		CP	12.2	-	-	-	-

unless the definitions of what constitutes a major and minor malformation are clearly defined. In the present study the definitions of major and minor anomalies used by Czeizel & Tusnady (1984) were adopted.

The present study has shown that a higher proportion of the CP group had associated abnormalities than the CL(P) group (YM x^2=15.54 p<0.001). This is in agreement with previous reports (Fraser 1970; Gorlin *et al.* 1976; Shprintzen *et al.* 1985). At least part of this difference is due to the relative frequency of Pierre Robin syndrome (PRS) which accounts for 23/86 (26.7%) of the CP group with associated abnormalities. If the isolated PRS group are removed from the calculation the excess of associated abnormalities in the CP group is not statistically significant (YM x^2=3.18 p<0.1 p>0.05).

In comparing data from the present study with those from the report of Shprintzen *et al.* (1985), there would appear to broadly similar rates of abnormalities in the CP groups (Table XVI) with some discrepancy between the associated abnormality rates in the CL(P) groups. There is also discrepancy in the aetiological sub-groups with a higher percentage of the Shprintzen group having single gene defects and a higher percentage of the West of Scotland patients having a chromosomal aberration (Table XVI). It would seem likely that these differences are due to the different methods of ascertainment used in the two studies, as the study of Shprintzen *et al.* (1985) is based on patients referred to the Center for Craniofacial Disorders in New York and is, therefore, likely to under-ascertain lethal multiple congenital abnormality syndromes (eg. trisomy 13) and, perhaps, over-estimate certain single gene defect as part of specialist referral policies (eg. Treacher-Collins syndrome).

In the present study, a significant proportion of facial cleft patients have associated abnormalities that cannot be identified as part of a specific syndrome or sequence. This has also been observed in previous reports (Rollnick & Pruzansky 1981; Shprintzen *et al.* 1985). This may suggest that there are many more rare malformation syndromes not yet delineated

that include facial clefting. However, large studies of well documented cases will be required to identify specific and new patterns of malformations. Other authors have suggested that the common occurrence of other malformations in this group is part of a generalised developmental instability (Adams & Niswander 1967; Fraser 1970; Czeizel & Tusnady 1984). Adam & Niswander (1967) advanced the theory that facial clefting may be one manifestation of what they termed 'developmental noise' to denote the asymmetry of paired organs caused by non-genetic factors. They suggested that developmental noise may result from a familial susceptability to a specific teratogen and that this should be reflected by an increased incidence of congenital anomalies in close relatives and evidence of instability in other paired organs in the index cases. Again, the published data give conflicting results, with two studies showing no increased incidence of malformation in first degree relatives of patients with facial clefts (Rank & Thomson 1960; Curtis *et al.* 1961) and two giving statistically significant increases (Drillien *et al.* 1966; Niswander & Adams 1968). There has been considerably more consensus on the presence of asymmetry in the index cases. This being demonstrated in familial CL(P) cases by the use of dermatoglyphic patterns (Adams & Niswander 1967) or dental impressions (Crawford & Sofaer 1987). It is of interest that this increase in asymmetry was not present in non-familial CL(P) or in any subgroup of CP, and thus may represent a minor role in the aetiology of this very heterogeneous group of patients.

In conclusion, it would seem that the data presented in this study are in broad agreement with previous reports of facial clefts in European populations. It is of interest, however. that there would appear to be a relative excess of isolated cleft palate in reports from this study and from previous reports from other more Northern regions of Europe which, in the absence of a demonstrable seasonal variation, may suggest a non-cyclic environmental agent contributing to aetiology. The present study would also appear to be one of the first reports to detail the number and nature of major congenital abnormalities associated with a completely ascertained population of facial clefts. Finally, this study has

established a database of patient data that can be used in future investigations to calculate empiric recurrence rates for family counselling and for association studies with restriction fragment length polymorphisms.

4.2 The RNA localisation studies

As the questions raised by clinical investigations of facial clefts become more defined, it is of increasing importance to understand the mechanisms controlling mammalian palatogenesis at the cellular and molecular level. The difficulty in obtaining non-traumatised human embryos of the appropriate developmental stages has led to the widespread use of animal models in the study of palatogenesis. The mouse represents a good model for such a study since the mechanisms of palate development are almost identical to those in the human, and the process can be disturbed in a controlled manner by the administration of teratogens (Morriss, 1973; Abbott *et al.* 1988). Furthermore, several genetic lines of mice are available which show increased susceptiblity to this malformation (Fitch, 1957; Gasser *et al.* 1981).

The genes involved in any disease process can be identified either directly by studying the expression patterns of candidate genes in normal and diseased tissue, or indirectly by linkage analysis or haplotype association. In this study the former approach was taken, using *in situ* hybridisation of anti-sense ribonucleotide probes to sectioned staged mouse embryos. The genes that were chosen for study were the transforming growth factor type ß, isoforms 1, 2 and 3 (TGFß1, TGFß2 and TGFß3). These genes are part of an extensive family of polypeptide multifunctional cell regulators (see section 1.) and have been suggested by Ferguson (1988) to be candidate genes in normal palate development on the basis of *in vitro* properties and immunohistochemical localisation (Heine *et al.* 1987).

The use of *in situ* hybridisation to cellular RNA has several advantages

over other RNA detection methods in the study of developmental processes. The first is the ability to analyse the tissue distribution of RNA species very precisely (Pardue, 1985). Detailed localisation studies are not possible using Northern blot or reverse transcription/polymerase chain reaction (RT/PCR) methods as these both require RNA to be extracted from the tissue. Another advantage that the *in situ* technique offers is its high degree of sensitivity (Bandtlow *et al.* 1987) with an ability to detect 10-20 copies of mRNA per cell. Although *in situ* hybridisation is not the most sensitive method of RNA detection (RT/PCR is capable of detecting a single RNA molecule) it would appear to be more sensitive than Northern blot analysis which has a limit of detection thought to be in the order of 10,000 mRNA molecules (Kawasaki 1990).

The technical limitations of *in situ* hybridisation are related to the inability to quantify the results in detail and the possibility of cross-hybridisation with similar RNA species. These problem have been minimised in the present study by careful control over the size and specific activity of the radioactive probe, the use species specific riboprobes from relatively non-homologous areas of the gene and highly stringent hybridisation conditions (see section 2.2).

In the RNA localisation studies presented below two assumptions are made. First that the gene-specific probes used in the *in situ* hybridisation recognise *bona fide* mRNA for the genes they represent. In a study using the same TGFß1 probe that was used in this study good correlation was found between the *in situ* data and RNA gel blot analysis (Akhurst *et* al.1988). Furthermore the gene-specific probes used in the present study do recognise transcripts of the predicted size for each of the isoforms (Denhez *et al.* 1990). The second assumption is that the RNA detected is translated into biologically active protein. This is complicated by the presence of post-transcriptional mechanisms that are known to control the production of biologically active TGFß, by limiting both protein secretion and activation of the latent form in the extracellular environment (Roberts & Sporn, 1990).

4.2.1 RNA localisation in normal mouse embryos

The stages of normal mouse palatogenesis can be summarised as appearance and vertical growth of the palatal shelves down the side of the tongue from 11.5 to 13.5 gestational days (GD), elevation of the shelves to the horizontal position (14.0 GD) with apposition and fusion of the shelves above the tongue (14.5 GD)(see section 1.). In the present study, *in situ* hybridisation was used to study the expression of TGFß genes during these critical stages of palatogenesis. The first evidence of the expression of these genes during murine palatogenesis is at 13.5 GD with epithelial expression of TGFß3 in the vertical palatal shelves and in the epithelium of the nasal septum that will go on to fuse with the palatal shelves. The palatal shelf epithelial expression of TGFß3 would appear to be restricted to the areas that will form the medial edge epithelium (MEE) of the palatal shelf after reorientation. This remarkable restriction of TGFß3 expression to the MEE is also seen after shelf elevation and continues in the epithelial seam that is formed from the MEE immediately after fusion, with loss of this epithelial expression as the epithelial seam disrupts by epithelial to mesenchymal transformation.

TGFß1 RNA shows a similar epithelial pattern of expression to that of TGFß3, but is not detectable in the MEE until after palatal shelf elevation and at this stage apparently has a lower transcript prevalence than TGFß3. As mentioned above the comparison of relative transcript levels of different genes by *in situ* hybridisation is difficult to make, despite control over the size and specific activity of the radioactive probe. Thus, although there would appear to be temporal differences between the expression patterns of TGFß1 and TGF ß3 these may simply reflect the quantitative limitations of the technique used. One conclusion that can be drawn regarding transcript prevalence is that the medial edge epithelial cells are the most abundant source of TGFß3 RNA within the murine embryo at this period of development (Millan *et* al. 1991).

The distribution of TGFß2 gene expression is distinct from those of

TGFß1 and TGFß3. TGFß2 RNA can first be detected in the hyperplastic nodules on the putative oral epithelia of the palatal shelves that will go on the form the palatal rugae (Sakamoto *et al.* 1989). After shelf elevation, however, TGFß2 expression is limited to the mesenchyme that underlies the MEE and epithelial seam. The temporal and spatial distribution of transcripts from these three genes is summarised in Figure 18.

After the results from the present study were published (FitzPatrick *et al.* 1990) a study from an independent laboratory, using *in situ* hybridisation of gene- and species-specific cRNA probes to sectioned mouse embryos reported identical RNA localisation results for TGFß1, TGFß2 and TGFß3 during palatogenesis (Pelton *et al.* 1990). As Pelton *et al.* (1990) used riboprobes complementary to different regions of the TGFß isoforms transcripts, their results confirm the different expression patterns of all three isoforms during palatogenesis.

Obviously knowledge of the distribution of the active protein product of the TGFß isoforms is necessary for an understanding of their mode of action in the palate. Several problems have, however been encountered in the localisation of these gene-products. The most widely used method of detecting tissue distributions of proteins is by using directly or indirectly labelled antibodies (for review see Harlow & Lane 1988). The generation of isoform-specific antibodies in the TGFß family has, however, proved very difficult with cross-reactivity between the isoforms often occurring in one assay but not in another (Flanders et *al.* 1988) and further complicated by the fact that antibodies raised to identical peptides can recognise different epitopes (Flanders *et al.* 1989). The problems encountered in raising specific antibodies to the TGFß family members are presumably related to the high degree of homology between the isoforms at an amino acid level and similar tertiary protein structure. Although there is almost the same degree of homology at a nucleotide level the ability of the investigator to design riboprobes that take advantage of the regions of relatively low homology between isoforms is not available to those working with antibodies.

Figure 18 Expression of TGFß Isoforms During Palatogenesis

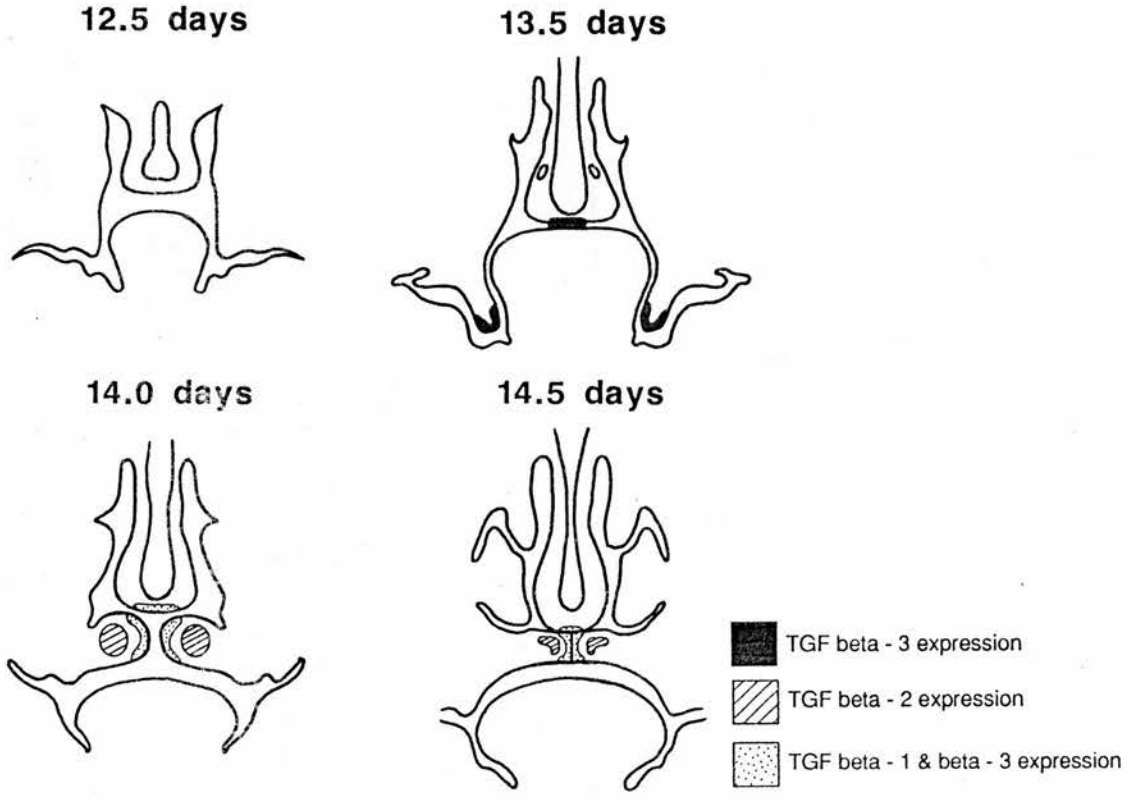


Figure 18. Diagrammatic representation of the role of the TGF beta family in the developing palate. The line drawings represent coronal sections through the midpalate at the gestations (in days) indicated above the drawings. The diagrams show the palatal shelves (ps) growing down beside the tongue (to) and then elevating to fuse between the tongue and nasal septum (ns). The filled black areas illustrates TGF beta3 expression, diagonal striping for TGF beta2 and stippled areas for the combined expression of TGF beta1 and beta3.

Heine *et al.* (1987) used a polyclonal antibody to study immunohistochemical localisation of TGFß1 polypeptide in the mouse embryo. This study showed TGFß1 to be present in the mesenchyme of the fusing palate. Although no detail was given of the spatial distribution within the palatal mesenchyme, these results are consistent with a paracrine mode of action of epithelially-derived TGF ß1, as previously suggested by Lehnert & Akhurst, (1988). This model for TGFß1 action was proposed after TGFß1 RNA had been localised to certain differentiating epithelia at sites undergoing morphogenesis (eg. whisker follicle, tooth bud) where TGFß1 polypeptide had previously been localised to the underlying mesenchyme (Heine *et al.* 1987). Lenhert & Akhurst (1988) suggested that a latent form of the peptide, which is not recognised by the polyclonal antibody used by Heine *et al.* (1987), is produced in the epithelial cells, transported to the underlying mesenchyme where it is proteolytically cleaved to its active form. It is also possible, however, that the antibody used in the localisation could be recognising a different gene (eg TGFß2). It will be difficult to resolve this dilemma until a more specific method of localising these proteins is available.

From the similar pattern of epithelial TGFß 3 gene expression one might speculate that this growth factor also has paracrine activities on the mesenchyme. However there have been no embryonic localisation studies of TGFß3 polypeptide reported as yet.

Assuming the paracrine mode of action, TGFß1 (and ß3?) may act on the underlying mesenchyme in a variety of ways. TGFß1 is known to stimulate differentiation, proliferation and chemotaxis of cells of mesenchymal origin (Moses *et al.* 1985; Robey *et al.* 1987; Postlethwaite *et al.* 1987). Sharpe and Ferguson (1988) however have shown that although transforming growth factor type alpha (TGF alpha), fibroblast growth factor (aFGF,bFGF) and platelet derived growth factor (PDGR) were potently mitogenic in cultured medial edge palatal mesenchyme (MEPM), TGFß1 consistantly inhibited MEPM cell proliferation at a concentration of 1 ng/ml. Since both cell migration and proliferation are

essential features of palatal shelf development these data suggest that TGFß1 may have a role in regulating cell growth in the palatal shelves. In this context it is interesting that the timing of TGF ß3 expression as a putative growth inhibitor in the vertical shelves corresponds to the period when their linear growth ceases (Ferguson 1987), it is also known however that this is the period of maximal mesenchymal proliferation judged by counting mitotic figures in colchicine-treated embryos (Jelinek & Dostal 1973) and [^{3}H]-thymidine incorporation (Hassel *et al.* 1974). It is therefore tempting to speculate that delineated areas of proliferation adjacent to areas of inhibition may have a role in the dramatic processes involved in shelf reorientation .

TGFß1 is also known to have a major effect in the elaboration of the extracellular matrix (ECM) components and on the synthesis of cell-surface receptors for ECM components (Heine *et al.* 1990). It is now widely accepted that the composition of the ECM can influence cell-to-cell and cell-to-substratum connections, which will result in cell migration or stabilisation of organ structure. This suggests that ECM molecules play an important role in embryogenesis, Nakanishi *et al.* (1986), for example, have shown that collagen type III is essential to cleft formation in the development of the salivary gland.

TGFß1 is known to induce the synthesis of collagens and fibronectin (Roberts *et al.* 1986; Ignotz *et al.* 1987), tenascin (Pearson *et al.* 1988) and chondroitin/dermatan proteoglycans (Hiraki *et al.* 1988; Sharpe & Ferguson, 1988). Accumulation of the latter class of molecules is thought to be important in palatal shelf elevation by virtue of the rise in osmotic pressure resulting from hydration of the proteoglycan network (Pratt *et al.* 1973; Brinkley & Morris Wiman, 1987; Derynck *et al.* 1988a). In this respect it is interesting that high levels of TGFß3 RNA are observed 24 hours prior to palatal shelf elevation.

The distributions of many extracellular matrix proteins are fairly ubiquitous within the palatal mesenchyme. Two significant exceptions are collagen IX and tenascin. Collagen IX appears on the cell surface of

medial edge epithelial cells prior to shelf elevation (Ferguson, 1988). Tenascin is localised beneath the medial edge epithelium prior to and during palatal shelf fusion (Sharpe & Ferguson, 1988). Since TGFß1 is known to induce synthesis of both of these proteins (Sharpe & Ferguson, 1988; Pearson *et al.* 1988), it is a reasonable supposition that these ECM molecules may mediate some of the effects of TGFßs. The distribution of tenascin is particularly significant since the embryonic distribution of this molecule is almost completely correlated with the prescence of epithelial TGFß1 RNA (Chiquet-Ehrismann *et* al. 1986; Lehnert & Akhurst, 1988; Akhurst *et al.* 1990; Sharpe & Ferguson, 1988).

Tenascin has two properties that strongly suggest a functional role in palatal shelf mesenchyme. The first is the ability of tenascin to disrupt epithelial sheet continuity by breaking cell-cell and cell-substratum contacts (Chiquet-Ehrismann *et al.* 1989). The second is its ability to promote specifically the mobility of neural crest cells *in vitro* (Halfter *et* al. 1989). Both of these events would be necessary for fusion along the mid-line seam, a time when the epithelial sheet disrupts, epithelial cells transdifferentiate to a mesenchymal cell phenotype and there is much cell mixing.

The processes involved in the disruption of the midline epithelial seam (MES) have been the subject of much interest over the last two decades. For most of this time the major mechanism in the disappearance of the epithelial cells was thought to be terminal differentiation, or "programmed cell death" with the removal of the cell remains by macrophages (Pratt & Martin, 1975; Greene & Pratt, 1976). Programmed cell death is a fascinating event in palatogenesis, with cessation of epithelial DNA synthesis estimated by the incorporation of [^{3}H]-thymidine occuring 24-36 hours prior to fusion (Pratt & Martin, 1975). It is accompanied by a down-regulation in epidermal growth factor (EGF) receptors (Abbott *et* al. 1988), and is not dependent on shelf contact in vitro. This lethal differentiation is specific to the medial edge epithelium; it is not seen in the oral or nasal components (Pratt & Martin, 1975; Tyler & Koch, 1975). However, little is known of the cellular

events that cause this autolysis. It is interesting that the activation of TGFß3 gene expression occurs 24 to 36 hours prior to fusion and could be important in this growth inhibitory process.

However, recently the work of Fitchett & Hay (1989) has convincingly shown that a second mechanism is involved in MES disruption. Using electron microscopy they have demonstrated transdifferentiation of the medial edge epithelial cells to mesenchymal phenotype and suggest that this may be the predominant process in the disruption of the MES, with programmed cell death resulting in peridermal sloughing before apposition of the shelves and formaton of the MES. Transdifferentiation has been shown to be important in the morphogenesis of several systems such as the thyroid gland (Greenburg & Hay 1988) and the regression of the Mullerian ducts (Cunha *et* al.1981); both of these processes would appear to be under careful developmental control. Little is known of the cell signals that induce transdifferentiation, however it is interesting to note that lens epithelia transform to mesenchymal phenotype when cultured within a collagen matrix (Greenburg & Hay 1982), which could point to a role for the TGFß family in this process.

TGFß1, TGFß2 and TGFß3 are each known to be growth inhibitory to epithelial cells, antagonising the mitogenic activities of TGF alpha and epidermal growth factor (EGF) (Massague, 1985; Like & Massague, 1986; Coffey *et al.* 1988; Grayar *et al.* 1989). It is however unlikely that EGF receptor down-regulation (Abbott *et al.* 1988) is directly initiated by TGFßs. Although this is a mechanism of negative growth regulation utilised by endothelial cells (Takehara *et al.* 1987), TGFß acts distally to the EGF receptor in growth inhibition of all epithelial cells that have been examined (Massague, 1985; Like & Massague, 1986; Coffey *et al.* 1988).

TGFß2 RNA distribution during palatogenesis is in marked contrast to that of TGFs ß1 and ß3. Its predominant localisation in the mesenchyme would agree with the observations of Pelton *et al.* (1989). It was suggested by Pelton *et al.* that mesenchymal expression of TGF ß2 might

be important, not only in modulating the mesenchyme *per se*, but in supporting growth of the overlying epithelium *via* secondary events such as induction of TGF alpha. In this context, it is interesting that the TGFß2 RNA distribution is asymmetric with respect to the nasal and oral regions. Differential concentrations of growth factors within the mesenchyme could contribute to the generation of regional heterogeneity of the overlying epithelium.

Recently Abbott & Birnbaum (1991) have reported immunohistochemical localisation of TGFß2 during murine palatogenesis using a commercial polyclonal antibody to porcine TGFß2. This antibody gave a uniform staining pattern over the epithelia and mesenchyme of the palatal shelves from 12 GD to fusion of the palate. This pattern is difficult to explain given the site- and stage-specific RNA localisation pattern shown by *in situ* hybridisation (FitzPatrick *et* al 1990; Pelton *et al.* 1990). The authors, however, suggest that the results are consistent with a paracrine mechanism of action for TGFß2 in the palatal shelf epithelia. Given the problems of producing isoform-specific antibodies mentioned above further studies will be required to confirm this protein localisation.

The localised high level expression of TGFß2 in the hyperplastic nodules of the early medial edge epithelium that represent the palatal rugae is consistent with this growth factor acting as an inducible homeostatic regulator of epithelial growth and differentiation. The role that the rugae play in the development of the palate is unclear, however, in their comprehensive investigation, Sakamoto *et al.* (1989) suggest that the 'stiffness' of the rugae may facilitate shelf elevation. Pelton *et al.* (1989) previously observed the expression of TGFß2 in the suprabasal keratinocytes of the embryonic skin at a time when the rate of keratinocyte cell division would be slowing.

One question raised by these RNA localisation studies is why genes encoding proteins with such similar *in vitro* biological activities should be expressed in the same developing organ, in some cases with

overlapping but distinct patterns of expression. One explanation of the apparent temporal sequence of expression (TGFß3 followed by TGFß1 and TGFß2) would be the induction of one member of the TGFß family by another, to amplify specific prefusion biological effects. It is known that TGFß1 positively regulates its own expression in normal and transformed cells (Van Obberghen-Schilling *et al.* 1988), but little is known about "cross-talk" between these individual genes. Clearly these different genes must serve some disparate *in vivo* biological functions. This is supported by the fact that some *in vitro* biological activities reported for the TGFßs do show some specificity in isoform requirement (Rosa *et al.* 1988; Jennings *et al.* 1988).

Further insight into the role of these isoforms may be gained by studying their localisation in the other developmental processes. During the period of this study several reports of TGFß isoform expression in embrogenesis were published (Akhurst *et al.* 1990; Gatherer *et al.* 1990; Millan *et al.* 1991; Pelton *et al.* 1990). Gatherer at *al.* (1990) have published the first extensive study of TGFß isoforms expression in mammalian embryogenesis using *in situ* hybridisation to early human embryos. This study suggested that the three isoforms are not generally expressed in the same developing systems. The only exception to this rule in the human embryo appears to be the developing lung where TGF ß2 RNA was seen in the primitive lung epithelia from around 43 GD; this is the period of proliferation and branching of the lung buds. TGF ß3, however, showed a different expression pattern, being found submucosally in the larger airways (bronchi and trachea) at around 57 GD. These results were supported by a similar pattern reported in the murine developing lung (Millan *et al.* 1991). Using mouse embryos Millan *et al.* (1991) were, however, also able to report concommitant expression of two or more of the TGFß isoforms in the epithelia of several developmental systems that could not be studied in the human embryos, such as the whisker follicles (ß1, ß2 and ß3 RNA detected) and the salivary gland and the tooth bud (ß1 and ß2). While no direct analogies can be drawn, these systems all represent structures that are actively involved in the process of morphogenesis and therefore display

some similarity to the developing palate.

It is also interesting that these localisation studies show that each of the TGFß isoforms are expressed epithelially at some point during murine and human embryogenesis. Specifically, the association of these isoforms with epithelia overlying active mesenchyme in developmental structures beyond the palate strengthen the conclusion from the present study that they have a major role in the development of the palate.

4.2.2 RNA localisation in retinoic acid treated embryos

A natural extension of the initial studies presented above was to repeat the *in situ* hybridisation studies on embryos that would go on to develop cleft palate. This could be done either by breeding from mice homozygous for a non-lethal recessive gene that causes clefting (eg Tw or P^{cp} mutants) or by treating pregnant mice from susceptible strains with the appropriate teratogenic agent. The latter approach was taken in the present study, employing all-trans retinoic acid-treated C57BL/6 mouse embryos. There were two reasons for this choice. First, the teratogenic effects of retinoic acid (RA) have been the subject of a great many studies (see section 1.1.3.2) and the doses required to induce cleft palate in this strain of mice have been published (Abbott *et al.* 1989). Secondly, RA has long been considered an important modulator of normal development (Sporn & Roberts, 1984) and recent *in vitro* studies have suggested that at least some of its morphogenic actions may be mediated *via* TGFß gene activation (Glick *et al.* 1989) .

Two sets of embryos were used in the experiments, those supplied as tissue sections by Dr. Barbara Abbott (NIH, Bethesda) and those treated and collected in Glasgow as part of the present study. Both sets of embryos were from C57BL/6 crosses, timed and collected in the same manner with identical fixation and embedding protocols. The major difference between the sets of embryos was the timing of the RA treatment (100mg/Kg), which in the Bethesda embryos was on 10.0 GD (with embryos collected on 12.5 GD and 14.5 GD) and in the Glasgow

was on 12.0 GD (embryos collected on 14.5 GD).

The C57B control embryos used in this part of the study gave TGFß isoform expression patterns which were identical to those seen in the NIH/Parkes embryos (see section 4.2.1). This suggests that the expression patterns are independent of the strain of mouse used, although further studies on different species will be required before this can be accepted as a general pattern in mammalian development. The phenotypic abnormalities in the RA-treated specimens were obvious on the embryos collected on both 12.5 GD and 14.5 GD with severe hypoplasia of the palatal shelves. However, the RNA expression patterns shown by the treated embryos on 14.5 GD were similar to those in the control embryos regardless of timing of treatment. TGFß1 and ß3 riboprobes hybridising to the MEE and TGFß2 RNA localised to the medial edge palatal mesenchyme.

Glick *et al.* (1989) have shown that RA treatment of cultured keratinocytes causes the induction of TGFß2 RNA expression, with subsequent secretion of biologically active peptide by the treated cells. This is the first direct evidence for involvement of the TGFß family in retinoid-induced mechanisms. It is tempting to speculate that the induction of TGFß2 may have a role in the hypoplasia of the palatal shelves in RA-treated embryos. Although no studies of the effect of TGF ß2 peptide on palatal mesenchyme has been published it is reasonable to assume that it would have the same potently growth-inhibitory effect as TGFß1 (Sharpe & Ferguson, 1988). The difficulty in quantification of results makes *in situ* hybridisation a poor technique for the study of up-regulation of gene transcription. It is possible, therefore, that although a normal expression pattern is seen in the RA-treated embryos in the present study more RNA is present than in the control embryos. Another possiblity is that normal amounts of RNA are present in the palatal shelves with post-translational regulation of TGFß2 activity. Abbott & Birnbaum (1991) have recently published a study of immunohistochemical localisation of TGFß1 and TGFß2 in retinoic acid-treated mice which has shown a significant increase in TGFß2

antibody staining in the nasal epithelial cells but not the subepithelial mesenchyme. The authors, therefore, suggest that TGFß2 gene is up-regulated with the secreted peptide acting on the nasal epithelium *via* a paracrine mode of action. Doubts over the specificity of antibodies to the TGFß isoforms have, however, been raised (see section 4.2.1) and evidence of up-regulation of the gene on RNA gel electrophoresis analysis is required to confirm this.

The cloning of the three retinoic acid receptors (RAR's) (Petkovich *et al.* 1987; Giguere *et al.* 1987; Brand *et al.* 1988; Zelent *et al.* 1989) has provided evidence that TGFß molecules may modulate RA action in ways other than *via* TGFß2 gene activation. The RAR's are members of the steroid hormone receptor superfamily (Zelent *et al.* 1989) and several recent studies have suggested that TGFß polypeptide acts as a local mediator of cellular responses to steroids and related hormones such as parathyroid hormone (Pfeilschifter & Mundy 1987) and oestrogen (Knabbe *et al.* 1987; Komm *et al.* 1988). Obviously, if the interaction of TGFßs and RAR's are important in the teratogenic effects of RA, then one would expect these genes to be co-expressed in or around the affected tissues during organogenesis. Unfortunately no studies of RAR expression during palatogenesis are available as yet. Dolle *et al.* (1990) have, however, studied the expression of RAR-alpha, -ß and gamma genes during murine organogenesis. Although RAR-alpha was found to be expressed almost ubiquitously, both RAR-ß (gut, bronchi, kidney, eye) and RAR-gamma (lung, oesophagus, endocardial cushions, skin) were found to have site- and stage- specific expression patterns. These localisations, however, do not correspond exactly to the embryonic expression patterns of TGF ß1 (Lenhert & Akhurst 1988), TGFß2 (Pelton *et al.* 1989; Gatherer et *al.* 1990; Millan *et al.* 1991) or TGFß3 (Gatherer *et al.* 1990; Pelton et *al.* 1990; Millan *et al.* 1991). Further detailed studies of individual systems in both normal and retinoic acid treated embryos are therefore required to clarify this fascinating area.

In conclusion the expression patterns of the TGFß isoforms are not

altered in retinoic acid-treated embryos that would go on to develop cleft palate. They must, however, remain candidate genes both as modulators of RA morphogenetic/teratogenic activity and important regulators of the normal processes involved in palatogenesis.

4.3 FUTURE PROSPECTS

4.3.1 Future directions for clinical investigation

The present study, like so many before it, would appear to have raised more questions than it has answered. However it must be hoped that establishing a well ascertained database of patients with facial clefts will provide a useful resource for clinical research. By collecting three-generation family trees from the families of the ascertained cases accurate empiric recurrence risk data can be calculated for use in genetic counselling. Furthermore, these family data can be used in complex segregation analysis to determine if there is evidence for the action of a major gene causing facial clefting in the West of Scotland. Chung et *al.* (1986) have reported that an autosomal recessive gene with low penetrance may be a significant cause of CL(P) in the Danish population. This last point has taken on particular significance with the reported association of a variation in the TGF alpha gene with non-syndromic cleft lip with or without cleft palate in a group of unrelated American patients (Ardinger *et al.* 1989). If confirmed in local populations, the association of a specific restriction fragment length polymorphism detected by a TGF alpha gene probe could be used to adjust the recurrence risk to the parents of a child with the 'low risk' haplotype.

Another intriguing avenue for clinical research has been suggestion that recurrences of facial clefts can be prevented by periconception multivitamin supplementation (Conway, 1958; Tolorova, 1982). Tolarova (1982) reported 1 recurrence out of 85 pregnancies (1.2%) in mothers with a previous child with CL(P) who were given periconceptual vitamin supplementation compared with 15/212 (7.4%) recurrences in the

control group. From the experience of vitamin supplementation trials in the prevention of neural tube defects a large multicentre trial would be required to confirm these findings.

A large number of patients with associated major anomalies which cannot be categorised have been shown in this and other studies of facial cleft populations. This fact must raise the possibility that a significant number of unrecognised syndromic entities are present within these groups. By pooling of data from several studies recognition of 'new' syndromes can be facilitated. To this end clinical details and photographs have been stored in all patients in the 'unknown' group in the present study.

4.3.2 Future directions for developmental biology

The present study has provided evidence for an important role for TGFß isoforms during normal murine palate development. Obviously, establishing the presence of biologically active peptide products for each of these genes is important, although this will not be possible until the sensitivity and specificity of immunohistochemical localisation of TGFß isoforms improves. At present the most exciting option in studying the role of these isoforms during palatogenesis is through transgenic technology. These techniques offer unparalleled opportunities to investigate the role of cloned genes in developmental processes. Although the most basic of these techniques (general overexpression and gene elimination studies) are unlikely to yield useful information in the study of palate development, the prospect of stage- and site-specific gene inactivation by using inducible promotor sequences which produce anti-sense transcripts may provide definitive answers to many of our questions.

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APPENDIX A

Single gene defects causing facial clefts

SYNDROME	MIM number	FEATURES
Dominant Phenotypes		
AASE-SMITH	147800	Hydrocephalus-CP-Joint contractures
ACROMICRIC DYSPLASIA	102370	Short limbs-Cleft lip-Distal limb abnormalities
ALLANSON [1985]	119580	Clefting-Ectropion-Teeth abnormalities-CL+/-P
APERT (Acrocephalo-syndactyly)	101200	Craniosynostosis-Prominent eyes-Syndactyly-CP
BLEPHAROPHIMOSIS	110100	Blepharophimosis-CP-MR-Simple ears
BRACHIAL PLEXUS NEURITIS + CP	162100	Short stature-CP-Peripheral neuropathy
CEREBRO-COSTO-MANDIBULAR	117650	IUGR-Gaps in ribs-Micrognathia-CP-MR
CLEFTING-ECTROPION-CONE TEETH	119580	CL(P)-Hypertelorism-Ectropion-Conical teeth
CLEFT LIP +/- CLEFT PALATE	119530	Isolated CL(P)
CL(P)/CP+FILIFORM FUSION EYELIDS	106250	CL+/-P-Synechiae-PDA
CLEFT LIP/PALATE-ECTRODACTYLY	129830	CL+/-P-Split hands
CLEFT PALATE	119540	Isolated cleft palate
CLEFT SOFT PALATE	119540	Isolated cleft of soft palate
CP-LATERAL ORAL SYNECHIAE	119550	Oral frenulae-CP-MR
DISTICHIASIS-LYMPHEDEMA	126320	Extra row of eyelashes-Lymphodema
ECTRODACTYLY-ED--CLEFTS-SCALP DEFECTS	129830	Split hands-Ectodermal dysplasia-CP
EEC	129900	Ectrodactyly-Ectodermal dysplasia-Clefting
FRONTONASAL DYSPLASIA	136760	Hypertelorism-Broad nasal tip
FUJIMOTO (Branchio-Oculo-Facial)	113620	Branchial clefts-Unusual facies
GORDON	114300	Clefting-Camptodactyly-Club foot
HAY-WELLS ECTODERMAL DYSPLASIA	106260	Ankyloblepharon-Ectodermal dysplasia-Clefting
HEMI FACIAL MICROSOMIA WITH RADIAL DEFECTS	141400	Facial asymmetry-Radial defects
HO [1975]	188740	CP-CDH-Absent tibia-Polydactyly
HYPOGLOSSIA-HYPODACTYLY	103300	Aglossia-Adactyly
JOHNSON [1983]	147770	Alopecia-Hypogonadism-Deafness-Anosmia
KABUKI MAKE-UP SYNDROME	147920	MR-Short stature-Unusual facies
KNIEST	156550	Metatropic dwarfism
LARSEN	150250	Congenital dislocations-Unusual facies
MCALISTER [1985]	?108720	Lethality-Macrocephaly-Short limbs-CP
MEIGE - LYMPHEDEMA PRAECOX	153200	Lymphedema-Arthritis
MYHRE [1981]	139210	Growth deficiency-Clefting-Retardation
NAGER	154400	Radial defects-Craniofacial anomalies
ODD OCULO-DENTO-DIGITAL	164200	Microphthalmos-Syndactyly-Dental anomalies
OSTEOPATHIA STRIATA WITH CRANIAL SCLEROSIS	166500	Osteopetrosis-Deafness
PFEIFFER [1988]	101600	Absent fibula/ulna-Oligodact-CL+/-P
PHILLIPS-GRIFFITHS	120400	Coloboma-Hand abnormalities-MR
POLYDACTYLY-CLEFT LIP	174300	Polydactyly-Cleft lip
POPLITEAL WEB	119500	CL-Lip pits-Popliteal pterygia
RAPP-HODGKIN ECTODERMAL DYSPLASIA	129400	Ectodermal dysplasia-Clefting
REISS [1986] (Distal Arthrogryposis Type II Plus)	108130	Contactures-CLP-Ptosis
ROBIN OLIGODACTYLY	172880	PRS-Oligodactyly
ROBINOW [FETAL FACE]	180700	Short stature-Characteristic face
SAETHRE-CHOTZEN [acrocephalosyndactyly Type III]	101400	Craniosynostosis-Ptosis-Prominent ear crus

APPENDIX A

Single gene defects causing facial clefts cont.

SILENGO [1986]	155200	Asymmetric crying facies-Microcephaly
SPONDYLOEPIPHYSEAL DYSPLASIA CONGENITA	120440	Short stature-CP
SPRENGEL ANOMALY + CP	184400	Spregel anomaly-CP
STICKLER HEREDITARY ARTHRO-OPHTHALMOPATHY	108300	Myopia-CP-Skeletal dysplasia
TREACHER-COLLINS	154500	Mandibulofacial dysostosis
VAN DER WOUDE	119300	CL+/-P-Lip pits
VELO-CARDIO-FACIAL	192430	CP-Congenital heart disease-Characteristic facies
WAARDENBURG	193500	Heterochromia of iris-Deafness
WEISSENBACHER-ZWEIMULLER	108300	Neonatal form of Stickler syndrome
WEYERS OLIGODACTYLY	193530	Oligodactyly-CP
Recessive phenotypes		
AASE	205600	Triphalangeal thumb-Congenital anemia-CL+/-P
ACROCALLOSAL	200990	Agenesis corpus callosum-MR-Hallux duplication-CP
ACROFACIAL DYSOSTOSIS WITH POST-AXIAL DEFECTS	263750	Deafness-Coloboma-Distal limb abnormalities-CL+/-P
AGNATHIA-HOLOPROSENCEPHALY	202650	Holoprosencephaly-CL+/-P-Small/absent mandible
ATELOSTEOGENESIS II	?256050	Rhisomelia-CP-Genital abnormalities
BARTSOCAS-PAPAS	263650	Popliteal pterygium-Severe AR form
BROSNAN [1980]	233430	XY Gonadal dysgenesis-CP-CHD-Ectoderm dysplasia
CAMPTOMELIC DYSPLASIA	211970	Campomelia-CP-Genital abn-Early death
CHRISTIAN [1971] ADDUCTED THUMBS SYNDROME	201550	Adducted thumbs-Contractures-MR
CLEFT LIP +/- CLEFT PALATE	215900	Isolated CL(P)
CLEFT PALATE AND OMPHALOCELE	258320	CP-Omphalocoele
DE LA CHAPELLE DYSPLASIA	256050	IUGR-Short limbs-Hypopl lung-Campomelia
DIASTROPHIC DYSPLASIA	222620	Rhisomelia-TEV-Hitchhiker thumb
FACIO-CARDIO-RENAL	227280	Ataxia-CHD-Renal anomalies
FARAG [1987]	271640	Spondyloepimetaphyseal dwarfism-Joint laxity
FIBROCHONDROGENESIS	228520	Rhisomelia-Narrow chest-Lethality
FRASER (Cryptophthalmos syndrome)	219000	Cryptophthalmos-Renal agenesis-Syndactyly
FRONTO-FACIO-NASAL DYSOSTOSIS	229400	Hypertelorism-Hypoplastic alae-CL+/-P
FRYNS	228950	Acral defects-Cloudy corneae-Diaphragmatic defects
GERMAN [1975]	231080	Hypotonia-MR-Unusual face
GORLIN [1971]	216300	CP-Oligodontia-Stapes fixation-Carpal anoms
HARPER [1967] (Seckel-like syndrome)	210600	CNS abn-Short stature-Unusual facies
HMC	239800	Hypertelorism-Microtia-Clefting
HOLOPROSENCEPHALY	236100	Holoprosencephaly-CL+/-P
HULTEN [1978]	242860	Immune deficiency-Centromeric instability
HUTTERITE MANDIBULOFACIAL DYSOSTOSIS	248390	Treacher-Collins like syndrome (AR)
HYDROLETHALUS	236680	Hydrocephalus-Hydramnios-Polydactyly
HYPOCHONDROGENESIS	200600	Deficient ossification lumbar vertebrae-Short limbs
INSLEY-ASTLEY	215150	Chondrodystrophy-Deafness (Marshall-Stickler like syndrome)
JARCHO-LEVIN	277300	Vertebral anomalies-Respiratory difficulties
JUBERG-HAYWARD	216100	Clefting-Radial defects-MR
KAPLAN [1988] (acro-cranio-facial-dysostosis)	201050	Acrocephaly-Proptosis-CP-Digital abn.
KAUFMAN-MCKUSICK	236700	Hydrometrocolpos-Polydactyly-CHD
LOWRY [1972]	218550	Craniostenosis-Fibular aplasia
MALPUECH [1983]	248340	MR-Growth failure-Clefting-Urogenital anoms
MARDEN-WALKER	248700	Blepharophimosis-Joint contractures-Hypotonia
MECKEL-GRUBER [dysencephalia splancnocystica]	249000	Encephalocoele-CP-Polydactyly-Renal cysts
MEGEPIPHYSEAL- EPI/METAPHYSEAL DYSPLASIA WITH HOMOCYSTINURIA	249230	MR-Deafness-Large epiphyses

APPENDIX A

Single gene defects causing facial clefts cont.

MICROCEPHALIC PRIMORDIAL DWARFISM TYPE 3	210730	IUGR-Alopecia-Microcephaly
MULTIPLE PTERYGIUM	265000	Multiple pterigia
MULTIPLE PTERYGIUM SYNDROME (Type Chen)	253920	Lethal-Multiple pterygia
NANCE-SWEENEY DWARFISM	215150	Chondrodystrophy-Deafness
NUCHAL BLEBS-LETHAL DYSPLASIA	257350	Cystic hygroma-Skeletal dysplasia
OCULO-PALATO-CEREBRAL	257910	Microcephaly-CP-Persistant hypertrophic primary vitrious
ODONTOTRICHOMELIC	273400	Tetramelia-Ectodermal dysplasia
OFD-2 ORO-FACIO-DIGITAL II [MOHR]	252100	Oral frenulae-Lobed tongue-Polysyndactyly
PALANT	260150	Cleft palate-Face and limb anomalies
PENA-SHOKEIR	208150	Multiple ankyloses-Pulmonary hypoplasia
PETERS'-PLUS	261540	Peters'anomaly-CL+/-P-MR-Ear anomalies
PSEUDODIASTROPHIC DYSPLASIA	263180	Severe skeletal dysplasia-Dislocations
RHIZOMELIC CHONDRODYSPLASIA PUNCTATA	215100	Stippled epiphyses-CP
RICHIERI-COSTA [1985]	201180	Acrofrontofacionasal dysostosis
RICHIERI-COSTA [1987]	275220	Tibial hemimelia-CL+/-P
ROBERTS [PSEUDOTHALIDOMIDE]	268300	Severe absence deficiencies-CP
ROLLAND-DESBUQUOIS	224400	Dyssegmental dysplasia
RUDIGER	268650	Abnormal face-Diaphragm, limb and gut anomalies
SCHNECKENBECKEN DYSPLASIA Lethal short-limbed dwarfism	269250	Short limbs-Snail-like pelvic bones
SEEMANOVA [1985]	251260	Microcephaly with immunodeficiency
SILVERMAN DYSSEGMENTAL DWARFISM	224400	Skeltal dysplasia-CP
SMITH-LEMLI-OPITZ TYPE II	268670	Polydactyly-CHD-Genital Abn-CP-Renal anomalies
SMITH-LEMLI-OPITZ [RSH]	270400	Short stature-Urogenital anomalies
VARADI [1980] (orofaciodigital like syndrome)	277170	Polydactyly-CP-MR
VERLOOVE-VANHORICK [1981]	215850	Bilobed lungs-Rhizomelia-CL+/-P-CHD
XTE	278780	Xeroderma-Talipes equinovarus-Enamel defects
ZUNICH [1983]	280000	Ichthyosis-CP-MR-Coloboma of retina

X-Linked phenotypes

CLEFT PALATE-ANKYLOGLOSSIA	303400	CP-Ankyloglossia
LENZ MICROPHTHALMIA	309800	Microphthalmia-Narrow chest-Double thumbs
MANZKE	302380	Cleft palate-Accessory metacarpal
MELNICK-NEEDLES OSTEODYSPLASTY	309350	Short stature-Prominent eyes
OFD-1 ORO-FACIO-DIGITAL [TYPE 1]	311200	Oral frenula-Syndactyly
OPD II OTO-PALATO-DIGITAL II FITCH	304120	Microcephaly-CP-Syndactyly
OPD OTO-PALATO-DIGITAL [TAYBI]	311300	Deafness-Characteristic facies-Tree frog toes
OPITZ-BBB	313600	Hypertelorism-Hypospadias
PALLISTER-W	311450	Clefting-MR-Skeletal defects
ROBIN SEQUENCE WITH PERSISTENCE OF LEFT SUPERIOR VENA CAVA	311900	PRS-Congenital heart defects-Talipes
TOLMIE [1987]	312150	X-linked lethal multiple pterygium
VASQUEZ [1979]	309590	X-linked hypogonadism-Gynecomastia-Obesity-MR
WILDERVANCK (cervicooculoacoustic syndrome)	314600	Cervical fusion-Duanes anomaly

APPENDIX B

Facial cleft syndromes of unknown aetiology

Syndrome	*Status*	*Features*
ABRUZZO [1977]	SR	Cleft palate-Coloboma-Radial synostosis
ACROCEPHALOSYNDACTYLY (Waardenburg type)	SR	Acrocephaly-Buphthalmos-CP-CHD-Syndactyly
AMNIOTIC BANDS	SPORADIC	Constriction rings-Brain abnormalities-CL+/-P
ANYANE YEBOA [1983]	SR ?AR	Clefting-Corneal opacity-MR
ATELENCEPHALY	??	Cerebral/cranial abn-Genital abn-CP
ATKIN [1984]	SR	Oculo-Cerebro-Acral,Roberts like + Ectrodactyly
BAETZ-GREENWALT [1983]	SPORADIC	Hypoplastic right heart-CP-Microcephaly
BIJLSMA [1980]	SR	MR-Cleft palate-Aqueductal stenosis-Contractures
BOWEN-ARMSTRONG	SR ?AR	Clefting-MR-Ectodermal dysplasia
CAMPTOMELIC VARIANT	SR	Campomelia-Fractures-Ankyloglossia superior
CHARGE ASSOCIATION	SPORADIC	Coloboma-CHD-Choanal atr-Ear abn-Short
CLEFT LIP AND PALATE WITH PITUITARY DEFICIENCY	SR	CL+/-P-Hypopituitarism
CLEFTING-COLOBOMA (choroid)-MR	SR ?AR	Craniostenosis-Coloboma-Clefting-MR
CLEFTING-PREMAXILLA AGENESIS	SR ?AD	Microcephaly-Agenesis premax-MR-CP
CRANE [1981]	SR ?AR	Clefting with skeletal anomalies
CUMMING [1986]	SR ?AR	Campomelia-Polycystic dysplasia-Polysplenia
CUTIS GYRATUM WITH ACANTHOSIS NIGRICANS	??	Unusual face-Gut abn-MR
DAVIS [1976]	SR ?AR	MR-Unusual facies-CP-Genital abn
DE LANGE SYNDROME	SPORADIC	IUGR-Microcephaly-Synophrys-Limb defects
FEINGOLD [1983]	SR	MR-Short stature-Cleft palate-Abnormal ears
FERNHOFF [1980]	SR	Short stature, IUGR-Malar/Mandibular hypoplasia
FH-UF	??	Femoral hypoplasia-Unusual facies
FINE [1983]	SR	Severe plagiocephaly, Megacornea- Cleft palate
FROSTER-ISKENIUS	SR ?AR	Contractures-Torticollis-Hyperthermia
GAREIS [1971]	SR	Short stature-Cleft palate
GOLDENHAR [Facio-Auriculo-Vertebral]	SPORADIC	Hemifacial microsomia-Epibulb. dermoid-Vert. abn
GRIX [1975]	SR	Craniofacial abnormalities-Osseous defects-MR
GUM/JAW FUSION	??	Syngnathia
HALAL [1983]	SR ?AD	Microcephaly-Cleft palate-Retinal pigmentation
HALL [1983]	SR	Hemangiomatous branchial clefts-Pseudo-clefts
HARTSFIELD [1984]	SR	Holoprosencephaly-Ectrodactyly-Cleft face
HASPESLAGH [1985]	SR	MR-Pterygia-Shortness
HERRMANN-PALLISTER-OPITZ	??	Craniostenosis-Limb anoms
HIRSCHSPRUNG-CP-MR	??	Hirschsprung-CP-MR
HOLZGREVE [1984]	SR	Renal agenesis-Cleft palate-Skeletal anoms
HOWARD-YOUNG	SR	Microcephaly-Clefting-Preaxial polydactyly
HUNTER [1987]	SR	Prenatal neuroaxonal dystrophy-Peripheral gangrene
IESHIMA [1986]	SR ?AR	Peculiar face-Deafness-Pseudohermaphrodite-MR
JOHNSON [1974]	SR	Glossopalatine ankylosis-Cataracts-Abnormal digits
KAUFMAN [1971]	SR	CHD-Vertebral and renal anomalies
KINGSTON [1982]	SR	Uveal coloboma-Clefting-MR.
KLIPPEL-FEIL	SPORADIC	Cervicovertebral fusion
KRIEBLE [1984]	SR	Blepharophimosis-Cleft palate-AD
LEWANDOWSKI [1980]	SR	Short stature-Multiple vertebral anomalies
LINDSTROM [1975]	SR	Short stature-MR-Unusual face
LOWRY-MACLEAN	SR	CHD-Diaphragmatic defect-MR-Clefting
LUX [1983]	SR	Cutis laxa-Cleft palate-Unusual facies

APPENDIX B

Facial cleft syndromes of unknown aetiology cont.

MADOKORO [1987]	SR	Microcephaly-Short palpebral fissures-Micrognathia
MARTSOLF [1977]	SR	Skeletal dysplasia-Polydactyly-Pierre Robin
MELNICK-NEEDLES-LIKE SYNDROME	SR	Short stature-Prominent eyes
MICHELS [1979]	SR	Clefting with ocular anomalies
MIRROR IMAGE EAR PLUS OTHER ANOMALIES	SR	Corneal dermoid-Duplication of ear
MOERMAN [1985]	SR	Lethal-Short limb-Brain anomalies
NAGER (with very severe limb defects)	SR	Severe limb defects-Craniofacial anomalies
OCULO-PITUITARY	SR	Similar to septo-optic dysplasia
PEARL [1984]	SR	Anotia-Facial weakness-CHD
PIEPKORN [1977]	SR	Short rib-Polydactyly
PILOTTO [1975]	SR	MR-Clefting-Vertebral defects
RANTA [1984]	?	Pierre Robin-Cleft lower lip
RICHIERI-COSTA [1983]	SR	Acrofacial dysostos-CL+/-P-Triphalangeal thumb
SALINAS [1976]	SR	Microcephaly-Atypical clefting
SANDERSON [1983]	SR	Proptosis-Abnl fisting-Robin assoc-Hypospadias
SAY [1975]	SR	Cleft palate-Small head-Large ears
SCHISIS ASSOCIATION	SPORADIC	CP-NTD-Diaphragmatic hernia
SPERBER [1986]	SR	Acalvaria-Holoprosencephaly-Facial dysmorphism
STEINFELD [1982]	SR	Holoprosencephaly and limb defects
TERUEL [1987]	SR	Absent abdominal musculature-Microphthal-Joint lax
TOLLNER [1981]	SR	Polydactyly and visceral anomalies
TRABOULSI [1984]	SR	Clefting-Ptosis
VICI [1988]	SR	Agen corpus callosum-Immunodef-Cataract-Hypopig
VOLAR NAILS	?	Volar nails-CP
VSR	?	Joint contractures-Craniofacial malformation
WALKER [1974]	SR	Treacher-Collins-Limb defects
WEAVER [1987]	SR	Craniosynostosis-Facial and limb defects
WEAVER-WILLIAMS	SR	Microcephaly-CP-MR-Thin
WOLTER [1971]	SR	Papilla nigra-Cleft palate- Extra thumb
WOON [1980]	SR	Craniosynostosis-Absent thumbs
YIM [1982]	SR	Amelia-Hydrocephalus-Iris coloboma-CL+/-P
YOLKEN [1976]	SR	Sh limbed dwarf-Bowed femurs-Metaphyseal irreg
YOUNG [1982]	SR	Keratoconus-Corneal opacity-CL+/-P-MR
ZADIK [1983]	SR	Hypothyroid-Dermoid cysts-Cleft palate-Dry skin
ZIMMER [1985]	SR	Tetra-amelia with multiple malformations
ZLOTOGORA [1987] CLEFT LIP	SR	CL-Syndactyly-Hair alterations

SR = Single case report only
?? = Several reported cases but inheritance pattern uncertain
?AR/?AD = Possibly autosomal recessive/autosomal dominant
Sporadic = Many cases with no evidence of mendelism

APPENDIX C

Stillborn Facial Cleft Cases Ascertained in Present Study

Study No.	Lip Diagnosis	Palate Diagnosis	Date of Birth	Sex	Health Board	Minor Assoc. Anomalies	Major Assoc. Anomalies	Details of Associated Anomalies
CL(P) cases								
PM1862	L4	04	01/01/80	F	GG	1	1	DYSPLASTIC EARS/SKELETAL DYSPLASIA
PM1647	M2	02	27/02/80	F	AC	0	2	NTD/MICROPHTHALMIA
UNK01	B4	04	01/05/80	F	GG	0	2	CNS MALFORMATION/CHD
PM2102	B4	04	01/01/81	M	FV	0	1	AQUEDUCT STENOSIS
UNK02	B4	04	25/01/81	M	GG	0	1	ANKYLOGLOSSIA
UNK03	B4	04	12/07/81	F	GG	0	1	CHD
PM2176	B4	04	25/09/81	M	LA	0	2	HYDROCEPHALUS/IMPERFORATE ANUS
UNK04	B4	04	04/12/81	M	GG	0	4	POLYDACTYLY/LUNG HYPOPLASIA
PM2363	B4	04	01/01/82	F	GG	0	4	NTD/ORAL HAMARTOMA/SKELETAL DYSPLASIA
PM2328	B4	04	26/01/82	F	GG	0	2	AGENESIS PREMAXILLA/CHD
PM2956	R4	04	06/09/83	M	GG	0	5	EXOMPHALOS/DIAPHRAGMATIC HERNIA
UNK05	B4	04	23/02/84	F	GG	0	3	SKULL ABNORMALITY/MICROCEPHALY
PM3428	B4	04	14/12/84	M	GG	0	4	MICROCEPHALY/POLYDACTYLY/CHD
CP cases								
PM1753	00	02	10/06/80	M	GG	0	2	MICROCEPHALY/IUGR
PM2127	00	02	31/07/81	M	LA	0	2	ANENCEPHALY/MALROTATION
PM2487	00	02	22/06/82	F	GG	0	3	CHD/HYDROCEPHALUS
PM2984	00	02	01/01/83	M	AC	2	1	CHD/RENAL DYSPLASIA/POLYDACTLY
PM2806	00	02	11/04/83	F	GG	0	1	SEVERE GROWTH RETARDATION

APPENDIX D

Deaths Among the Facial Cleft Cases Ascertained in the Present Study

Study No.	Lip Diagnosis	Palate Diagnosis	Date of Birth	Sex	Health Board	Minor Assoc. Anomalies	Major Assoc. Anomalies	Details of Associated Anomalies
CL(P) cases								
PM1787	B4	04	19/10/81	F	GG	0	3	MICROCEPHALY/POLYDACTYLY/CHD
268402	R1	02	09/11/81	F	GG	0	3	MICROPHTHALMIA/SCOLIOSIS/CONTRACTURES
269237	R4	04	14/01/82	F	AA	0	3	CHD/MR
273558	B4	04	09/06/82	M	DG	0	4	CHD/ECTRODACTYLY/ANKYLOGLOSSIA
282796	B4	04	04/02/83	F	LA	0	4	DIAPHRAGMATIC HERNIA/CHD
284446	L3	04	19/06/83	F	AC	1	0	IUGR
UNK06	B4	04	07/11/83	F	GG	1	3	HYPERTELORISM/CDH
12584	L4	00	13/06/84	F	AC	0	3	DIAPHRAGMATIC HERNIA/CDH
299029	B4	04	11/07/84	F	GG	0	3	CHD/MR
305366	L4	04	04/08/84	M	FV	1	0	IRIS COLOBOMA
CP cases								
UNK07	00	03	18/02/80	F	GG	0	3	HYDROCEPHALUS/ANKYLOGLOSSIA
MG3245	00	03	30/04/80	F	GG	0	3	CHD/GENITAL ANOMALIES/IUGR
PM2077	00	02	08/06/81	F	GG	0	3	SEX REVERSAL/FEMORAL BOWING
267688	00	03	24/07/81	M	GG	0	1	SEVERE MAXILLARY HYPOPLASIA
12292	00	03	19/10/81	M	LA	0	1	MICROGNATHIA/TRACHEAL STENOSIS
UNK08	00	03	28/03/82	F	GG	0	3	DIAPHRAGMATIC HERNIA/CHD
MG3277	00	03	02/04/82	M	GG	0	4	RADIAL APLASIA/CHD/RENAL DYSPLASIA
UNK09	00	03	07/07/82	M	GG	1	1	CHD/MICROGNATHIA
276102	00	03	01/09/82	M	AC	1	4	CHD/GENITAL ANOMALIES/MICROPHTHALMIA
277443	00	03	05/11/82	M	GG	0	3	CHD/RENAL DYSPLASIA/LARYNGEAL STENOSIS
MG4488	00	02	13/12/82	F	GG	0	2	NTD
MG4448	00	03	07/03/83	F	LA	0	3	FEMORAL BOWING/CHD
290831	00	03	04/01/84	M	GG	0	4	MICROPHTHALMIA/COLONIC ATRESIA
290851	00	02	04/01/84	F	GG	1	2	CHD/TEV/WEBBING OF NECK

Appendix E

Facial cleft patients (excluding stillbirths and fatalities) ascertained in present study

Study No.	Lip Diagn.	Palate Diagn.	Date of Birth	Sex	Health Board	Minor Assoc. Anomalies	Major Assoc. Anomalies	Details of Associated Anomalies
CL(P) patients								
55940	R2	00	11/01/80	M	AA	0	0	NONE
378078	L2	00	18/01/80	M	GG	0	0	NONE
56405	B4	04	22/02/80	M	AA	0	0	NONE
336115	B4	04	24/03/80	M	GG	0	0	NONE
249590	L4	00	04/04/80	M	GG	0	0	NONE
CSA01	U?	??	07/04/80	F	AC	0	0	NONE
56949	L4	04	13/04/80	F	AA	0	0	NONE
251065	L4	04	21/04/80	M	LA	0	0	NONE
266566	R4	04	23/04/80	M	AA	1	2	MR/SYNDACTYLY/ANAL PIT
251153	R4	00	24/04/80	M	GG	1	0	PREAURICULAR PITS
58023	R2	00	03/05/80	M	DG	0	0	NONE
250642	R3	00	05/05/80	F	GG	1	1	LIP PITS/CDH
251781	R2	00	27/06/80	F	GG	0	0	NONE
57774	L4	04	29/06/80	M	DG	0	0	NONE
UNK10	U?	00	21/07/80	M	FV	0	0	NONE
254101	B4	04	02/08/80	M	LA	0	0	NONE
253434	B4	04	04/08/80	M	GG	0	0	NONE
254597	L4	04	22/08/80	M	LA	0	0	NONE
254623	L2	03	28/08/80	F	AC	0	0	NONE
58558	L4	04	23/09/80	M	AA	2	0	PTOSIS/LOW SET EARS
252835	B4	00	20/10/80	M	LA	0	0	NONE
256095	L3	04	09/11/80	M	AC	0	0	NONE
59274	L4	04	04/12/80	M	LA	0	0	NONE
CSA02	U?	00	07/12/80	F	GG	0	0	NONE
257358	L4	03	31/12/80	M	LA	0	0	NONE
257999	L2	00	03/01/81	M	AC	0	1	IUGR
257792	L2	00	08/01/81	M	LA	1	0	CYST LEFT EAR
59648	B3	00	17/01/81	M	AA	0	0	NONE
269884	B4	04	12/02/81	M	FV	0	0	NONE
260650	B4	04	11/03/81	M	GG	0	0	NONE
260136	L2	02	25/03/81	M	GG	0	0	NONE
327541	B3	03	04/06/81	F	LA	0	0	NONE
263565	L2	00	08/07/81	M	GG	0	0	NONE
263573	L2	00	14/07/81	M	GG	0	0	NONE
CSA03	U?	00	23/07/81	M	GG	0	0	NONE
266859	R3	00	30/07/81	F	LA	0	0	NONE
265812	R1	00	07/08/81	F	GG	0	0	NONE
265283	R4	04	15/08/81	M	AC	0	0	NONE
265820	B3	04	17/09/81	F	GG	2	0	STRABISMUS/HYPERMETROPIA
267638	L3	00	21/09/81	M	FV	0	0	NONE
266165	R4	04	27/09/81	F	AC	0	0	NONE
266017	R2	00	11/10/81	M	LA	0	0	NONE
62519	L4	04	12/10/81	M	AA	0	0	NONE
266878	L3	00	14/10/81	M	AC	0	0	NONE
266064	L4	04	15/10/81	M	LA	0	0	NONE
266794	B4	00	20/10/81	F	GG	2	0	DUPLICATED HALLUX
268632	B4	04	01/12/81	M	LA	0	0	NONE
268572	U?	00	10/12/81	M	LA	0	0	NONE
270687	R3	03	27/12/81	M	GG	0	0	NONE
268904	R4	04	07/01/82	F	LA	0	1	DYSPLASIC EAR
278784	R4	04	15/01/82	M	AC	0	0	NONE
268951	R4	04	22/01/82	F	AC	0	0	NONE
280963	L2	00	03/02/82	M	LA	0	0	NONE
270576	L4	04	23/02/82	F	AA	0	5	MR/ASYMMETRY/VERTEBRAL ANOMS
272128	L4	04	01/03/82	M	GG	0	0	NONE
65010	B2	00	06/03/82	M	AA	0	0	NONE
276582	L3	00	20/03/82	M	GG	0	0	NONE
66530	B2	01	12/04/82	M	AA	0	0	NONE
273425	L4	04	17/05/82	M	GG	0	0	NONE
273372	L4	00	01/06/82	M	GG	0	0	NONE
65990	L4	04	01/06/82	M	AA	0	0	NONE
275162	L4	04	17/06/82	F	GG	1	0	LIP PITS
274635	L4	04	22/06/82	M	GG	0	0	NONE

Appendix E

Facial cleft patients (excluding stillbirths and fatalities) ascertained in present study

Study No.	Lip Diagn.	Palate Diagn.	Date of Birth	Sex	Health Board	Minor Assoc. Anomalies	Major Assoc. Anomalies	Details of Associated Anomalies
CL(P) patients cont.								
274579	B3	00	30/06/82	F	AC	0	0	NONE
277011	B4	SM	25/07/82	M	FV	1	0	NEUTROPAENIA
CSA04	U?	00	22/08/82	M	GG	0	0	NONE
277801	R2	00	10/11/82	M	AC	0	0	NONE
279570	B4	04	07/12/82	M	GG	0	2	FALLOT/POSTERIOR FOSSA CYST
278755	L3	00	15/12/82	M	LA	0	0	NONE
281659	L4	03	12/01/83	M	GG	1	1	CONSTIPATION/NTD
282569	R4	04	10/03/83	M	DG	0	0	NONE
281697	L4	04	22/03/83	M	GG	1	2	MR/SHORT STATURE
282311	B3	04	03/04/83	F	AC	0	0	NONE
282547	B4	04	08/04/83	M	LA	0	0	NONE
297937	L4	04	10/04/83	M	LA	0	0	NONE
282665	L4	00	11/04/83	M	GG	0	0	NONE
284331	L4	04	17/04/83	F	FV	0	0	NONE
287175	L4	04	05/05/83	F	AA	0	1	HYDROCEPHALUS
CSA05	U?	00	13/05/83	M	AA	0	1	CHD
296097	B4	03	17/05/83	M	LA	0	0	NONE
412419	R3	00	08/06/83	M	GG	0	0	NONE
287850	B4	04	29/06/83	F	DG	0	0	NONE
285510	R4	04	19/07/83	M	LA	0	0	NONE
139954	U?	00	02/09/83	M	AC	0	0	NONE
289816	L2	00	23/09/83	M	AC	3	0	DYSPLASTIC EAR/SYNDACTYLY
288225	L3	00	27/09/83	F	AC	0	0	NONE
289817	B4	04	02/10/83	M	DG	2	0	ANKBLEPHARON/LIP PITS
291327	L3	00	06/10/83	M	FV	0	0	NONE
282556	L4	04	21/10/83	F	LA	0	0	NONE
71338	B4	04	21/10/83	F	AA	0	0	NONE
293025	R4	04	02/11/83	F	FV	0	0	NONE
CSA06	U?	04	02/11/83	M	GG	0	1	CHD
291418	B3	00	08/12/83	F	AC	0	0	NONE
290346	L2	02	14/12/83	F	LA	0	0	CHD
291167	B4	04	10/01/84	M	LA	0	0	NONE
291350	R4	04	04/02/84	F	DG	0	3	CHOLEDOCHAL CYST/RENAL ANOM
293042	L4	04	26/02/84	F	GG	0	0	NONE
295181	L1	00	22/03/84	M	DG	0	0	NONE
72454	L2	00	29/03/84	F	AA	0	0	NONE
72464	B4	04	02/04/84	F	AA	0	2	ANKBLEPHARON/ECTODERMAL DYSPL.
294616	M1	00	04/04/84	M	GG	1	1	HYPERTELORISM/MR
305365	L2	03	14/07/84	M	FV	0	0	NONE
298520	R2	00	23/07/84	M	AC	0	0	NONE
299131	L1	00	12/08/84	M	AC	0	0	NONE
300467	L3	00	31/08/84	F	GG	0	0	NONE
301385	L3	00	06/09/84	M	GG	0	0	NONE
300133	R3	03	09/09/84	M	GG	0	0	NONE
73682	L4	04	12/09/84	F	AA	0	0	NONE
299954	L1	00	16/09/84	M	GG	0	0	NONE
302309	B4	04	23/10/84	M	GG	0	0	NONE
305177	R3	03	26/10/84	M	FV	0	0	NONE
CSA07	U?	00	30/10/84	M	DG	0	0	NONE
64815	R2	00	25/11/84	F	AA	0	0	NONE
302355	R2	00	26/11/84	M	GG	1	0	RECTAL POLYP
303255	L3	03	05/12/84	M	AC	0	0	NONE
303482	R2	00	28/12/84	M	GG	0	0	NONE

Appendix E

Facial cleft patients (excluding stillbirths and fatalities) ascertained in present study

Study No.	Lip Diagn.	Palate Diagn.	Date of Birth	Sex	Health Board	Minor Assoc. Anomalies	Major Assoc. Anomalies	Details of Associated Anomalies
CP patients								
248031	00	02	02/01/80	M	DG	0	0	NONE
CSA08	00	03	05/01/80	F	GG	0	3	CHD/HYDROCEPHALUS/MICTOTIA
246856	00	02	07/01/80	F	LA	0	0	NONE
248032	00	03	23/01/80	F	GG	1	0	STRABISMUS
UNK14	00	01	03/02/80	M	GG	0	0	NONE
244055	00	03	02/03/80	F	GG	0	1	MR
UNK15	00	03	02/03/80	M	GG	1	1	ANKGLOSSIA/CHD
249463	00	02	20/03/80	M	AC	0	2	SYNDACTYLY/ECTODERMAL DYSPL
250643	00	02	07/05/80	F	GG	1	0	MICROGNATHIA
253688	00	01	07/05/80	F	GG	0	2	MR/ATAXIA
251126	00	02	16/05/80	F	LA	0	0	NONE
252449	00	03	05/06/80	F	AC	0	0	NONE
251740	00	03	11/06/80	M	GG	1	0	MICROGNATHIA
57700	00	02	20/06/80	F	AA	1	0	MICROGNATHIA
254478	00	02	06/07/80	M	GG	0	0	NONE
256030	00	02	12/07/80	F	AA	0	0	NONE
269067	00	02	20/07/80	M	LA	0	3	MICROTIA/VERTEBRAL ANOMALIES
252781	00	03	21/07/80	M	GG	2	0	INGUINAL HERNIA/SACRAL DIMPLE
255336	00	02	18/08/80	F	LA	0	3	SCOLIOSIS/JAW CYST/SHORT STATURE
299133	00	02	25/08/80	F	AC	0	1	CHD
255628	00	02	04/09/80	F	DG	0	0	MICROGNATHIA
255394	00	03	22/09/80	F	LA	1	0	MICROGNATHIA
328968	00	03	29/09/80	F	GG	0	0	NONE
256466	00	02	28/11/80	F	GG	0	0	NONE
257817	00	03	23/12/80	F	LA	0	0	NONE
327540	00	02	14/01/81	F	LA	0	0	NONE
258510	00	02	31/01/81	M	GG	0	0	NONE
269195	00	03	04/02/81	F	GG	0	0	NONE
259264	00	03	21/02/81	F	GG	1	1	STRABISMUS/SHORT STATURE
279724	00	01	07/03/81	F	AC	1	0	MICROGNATHIA
260537	00	02	11/03/81	M	DG	1	1	TEV/MR
260541	00	01	18/03/81	M	AC	0	0	NONE
261453	00	02	06/04/81	F	LA	2	0	SYNDACTYLY/ANKYLOGLOSSIA
262151	00	01	30/04/81	F	LA	0	0	NONE
269880	00	02	30/04/81	M	FV	0	0	NONE
262139	00	02	06/05/81	M	LA	0	0	NONE
261833	00	02	08/05/81	M	AC	0	2	CHD/NTD
263186	00	02	27/05/81	F	GG	0	0	NONE
263183	00	02	01/06/81	M	AC	0	0	NONE
263553	00	03	02/06/81	M	LA	0	1	SEVERE MR
263380	00	02	11/06/81	M	LA	2	0	INGUINAL HERNIA/UNDECENDED TESTES
264126	00	02	13/07/81	F	GG	1	0	MICROGNATHIA
61751	00	03	13/07/81	M	AA	0	0	NONE
62198	00	02	06/08/81	M	AA	0	0	NONE
62304	00	03	23/08/81	F	AA	1	0	MICROGNATHIA
265420	00	03	10/09/81	F	GG	0	0	NONE
265699	00	03	19/09/81	F	LA	0	0	TWIN
266233	00	03	18/10/81	M	LA	1	0	INGUINAL HERNIA
62964	00	03	09/11/81	F	AA	1	0	MICROGNATHIA
266949	00	01	15/11/81	F	GG	0	0	NONE
UNK17	00	03	26/11/81	F	GG	0	0	NONE
270685	00	02	22/02/82	M	GG	0	0	NONE
270978	00	01	28/02/82	F	AC	0	2	ACHALASIA/MR
272808	00	03	06/04/82	F	LA	0	0	NONE
281837	00	01	15/04/82	M	FV	0	0	NONE
274580	00	03	16/04/82	M	AA	1	2	CHD/HYDONEPHOSIS/INGUINAL HERNIA
273957	00	03	23/04/82	F	GG	1	0	INGUINAL HERNIA
273420	00	02	05/06/82	M	AC	0	0	NONE
237322	00	03	21/07/82	F	GG	1	1	CHD/DYSPLASTIC EAR
276151	00	02	07/08/82	F	GG	0	1	MILD MR
276502	00	01	25/08/82	M	AC	0	0	NONE
288309	00	02	13/09/82	F	GG	0	0	NONE
277078	00	02	23/09/82	M	GG	1	0	MICROGNATHIA
275375	00	02	24/09/82	M	AC	1	0	ECTOPIC TESTIS
276902	00	02	01/10/82	F	GG	1	0	MICROGNATHIA

Appendix E

Facial cleft patients (excluding stillbirths and fatalities) ascertained in present study

Study No.	Lip Diagn.	Palate Diagn.	Date of Birth	Sex	Health Board	Minor Assoc. Anomalies	Major Assoc. Anomalies	Details of Associated Anomalies
CP patients cont.								
277540	00	01	09/10/82	F	AC	0	0	NONE
278609	00	01	04/12/82	F	GG	0	0	NONE
280171	00	02	05/12/82	M	GG	0	2	SKELETAL DYSPLASIA/ASTIGHATISM
278890	00	02	11/12/82	M	AC	0	0	NONE
279685	00	03	12/12/82	M	GG	0	1	SHORT STATURE
280200	00	02	19/12/82	F	GG	1	0	STRABISMUS
68656	00	03	23/12/82	F	AA	0	1	MR
UNK19	00	02	10/01/83	F	GG	0	0	NONE
279724	00	02	22/01/83	M	AC	1	0	MICROGNATHIA/MYOPIA
279893	00	03	23/01/83	F	AA	0	0	NONE
295216	00	03	10/02/83	F	GG	0	0	NONE
69256	00	02	14/03/83	M	AA	1	1	HYPERTELORISM/COLOBOMA
CSA11	00	03	14/03/83	M	FV	0	0	NONE
282228	00	03	07/04/83	F	LA	0	0	NONE
UNK20	00	02	16/04/83	F	GG	1	1	MICROGNATHIA/SKIN ABNORMAL
296027	00	02	23/04/83	M	FV	0	0	NONE
69812	00	03	25/04/83	M	AA	1	0	MICROGNATHIA
UNK21	00	02	28/05/83	M	GG	0	3	CHD/PULMONARY HYPOPLASIA/GENITAL ANOM
70928	00	02	05/08/83	F	DG	0	0	NONE
293112	00	03	12/08/83	F	GG	1	1	CHD/HYOPIA
287121	00	02	22/08/83	F	GG	0	0	NONE
CSA12	00	03	28/08/83	M	GG	0	0	NONE
287305	00	01	05/09/83	F	LA	0	0	NONE
71358	00	02	07/10/83	M	AA	0	1	CHD
71356	00	03	09/10/83	F	AA	0	1	SCOLIOSIS
292011	00	03	11/10/83	M	LA	1	1	PREMATURITY/INGUINAL HERNIA
290677	00	02	09/11/83	F	GG	0	0	NONE
324384	00	02	16/11/83	F	AC	1	0	MIDFACE HYPOPLASIA
290423	00	03	19/12/83	M	LA	0	1	CHD
291271	00	02	20/12/83	F	AA	0	0	NONE
305174	00	02	31/12/83	M	FV	0	0	NONE
294515	00	03	07/01/84	M	GG	0	1	CHD
72367	00	03	04/02/84	F	AA	0	0	CHD/MR/TEV
294167	00	02	02/03/84	M	DG	0	0	NONE
294521	00	02	15/03/84	M	GG	0	4	FITS/MR
294177	00	02	06/04/84	F	AC	0	1	MICROTIA
294338	00	02	09/04/84	F	GG	0	0	NONE
295174	00	02	13/04/84	M	LA	0	0	NONE
296065	00	02	16/04/84	M	GG	0	3	MICROTIA/SHORT STATURE
73822	00	04	25/05/84	F	AA	0	0	NONE
298258	00	02	06/06/84	F	AC	1	0	FH LEBERS
UNK22	00	02	07/06/84	F	GG	0	2	NTD/TEV
297497	00	03	05/07/84	M	GG	0	0	NONE
MG3994	00	03	08/07/84	F	GG	0	4	MICROCEPHALY/POLYDACTYLY
323128	00	03	20/07/84	F	AC	0	0	NONE
CSA14	00	03	25/07/84	F	FV	0	2	CHD/CNS MALFORMATION
298974	00	03	27/07/84	F	LA	1	0	PTOSIS
299133	00	02	31/07/84	M	GG	0	1	SHORT STATURE
298434	00	03	06/08/84	M	GG	0	0	NONE
299118	00	02	09/08/84	M	GG	1	1	ASTHMA SHORT STATURE
73591	00	03	10/08/84	F	AA	1	0	MICRONGATHIA
298718	00	03	15/08/84	M	LA	1	3	NTD/CDH/UNDECENDED TESTIS
301141	00	01	05/09/84	F	DG	0	0	NONE
299884	00	03	06/09/84	F	LA	0	0	NONE
73821	00	02	11/09/84	F	AA	0	0	NONE
301284	00	02	12/10/84	F	AC	1	0	UMBILICAL HERNIA
302706	00	02	18/10/84	M	GG	0	0	NONE
302511	00	01	29/10/84	M	LA	0	0	NONE
64919	00	03	15/11/84	F	AA	1	0	MICROGNATHIA
302697	00	03	20/11/84	F	AC	0	0	NONE
303663	00	02	18/12/84	M	GG	0	0	ADOPTED
303571	00	03	26/12/84	M	LA	0	0	NONE
303367	00	03	31/12/84	M	GG	1	1	CHD

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Differential expression of TGF beta isoforms in murine palatogenesis

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Summary

We have studied the expression of genes encoding transforming growth factors (TGFs) beta1, beta2 and beta3 during development of the secondary palate in the mouse from 11.5 to 15.5 days *postcoitum* using *in situ* hybridisation. The RNA detected at the earliest developmental stage is TGF beta3, which is localised in the epithelial component of the vertical palatal shelf. This expression continues in the horizontal palatal shelf, predominantly in the medial edge epithelium, and is lost as the epithelial seam disrupts, soon after palatal shelf fusion. TGF beta1 RNA is expressed with the same epithelial pattern as TGF beta3, but is not detectable until the horizontal palatal shelf stage. TGF beta2 RNA is localised to the palatal mesenchyme underlying the medial edge epithelia in the horizontal shelves and in the early postfusion palate. The temporal and spatial distribution of TGF beta1, beta2 and beta3 RNAs in the developing palate, together with a knowledge of *in vitro* TGF beta biological activities, suggests an important role for TGF beta isoforms in this developmental process.

Key words: TGF beta, *in situ* hybridisation, mouse embryo, palatogenesis.

Introduction

The murine secondary palate arises from the bilateral maxillary processes of the head. These structures are composed of migratory neural crest cells associated with craniopharyngeal ectoderm (for reviews see Greene and Pratt (1976) and Ferguson (1988)). Mesenchymal cell proliferation within the maxillary processes results in the appearance of primordia of the palatal shelves (Burdett *et al.* 1988), which grow vertically down beside the tongue. A rapid elevation of the palatal shelves brings the two processes into horizontal apposition above the tongue. This is followed by the fusion of the medial edges of the palatal shelves resulting in a continuous palate which later undergoes chondrification and ossification in its anterior portion (Greene and Pratt, 1976; Ferguson, 1988).

The epithelia that cover the palatal shelves are regionally heterogeneous. The oral, nasal and medial edge epithelia can be identified by position, morphology and cell surface markers (Ferguson, 1988). They also have different cell fates. Whereas the nasal and oral epithelia differentiate into pseudostratified and squamous epithelia, respectively, the medial edge epithelium is destined to disrupt by a combination of epithelial cell death and epithelial–mesenchymal transformation (Fitchett and Hay, 1989). Palatogenesis occurs comparatively late in embryogenesis (fusion at gestation day 14.5 in the mouse), making it an easily amenable model for studying several developmental processes, most notably epithelial–mesenchymal interactions.

The importance of epithelial–mesenchymal interactions in the developing palate was established by the tissue recombination studies of (Ferguson and Honig, 1984), who showed that the regional specification of the palatal epithelium is controlled by the underlying mesenchyme. Little is known of any reciprocal signalling from the palatal epithelium. Recently, a paracrine mechanism of transforming growth factor beta1 (TGF beta1) action was proposed in certain epithelial–mesenchymal interactions, as suggested by localisation of TGF beta1 RNA in epithelia which overlie mesenchymal tissue containing the TGF beta1 protein (Heine *et al.* 1987; Lehnert and Akhurst, 1988; Akhurst *et al.* 1990*b*). To extend this study further, particularly with respect to mammalian palatogenesis, we examined the distribution of RNAs encoding TGF beta1, beta2 and beta3, from the time of appearance of the palatal processes, at 11.5 days gestation, to completion of palatogenesis at 15.5 days *post-coitum*.

The TGF betas are a family of related polypeptides which show a high degree (65–80%) amino acid homology within the mature *C*-terminal portion of the molecule and are highly conserved throughout evolution (Sporn *et al.* 1986; Roberts and Sporn, 1990).

There are currently five vertebrate members of this family termed TGF beta1 (Derynck *et al.* 1985), TGF beta2 (Madisen *et al.* 1988; Miller *et al.* 1989*b*), TGF beta3 (ten-Dijke *et al.* 1988; Derynck *et al.* 1988; Jakowlew *et al.* 1988*b*; Denhez *et al.* 1990; Miller *et al.* 1989*a*), TGF beta4 (Jakowlew *et al.* 1988*a*) and TGF beta5 (Kondaiah *et al.* 1990), though only three have been molecularly cloned from mammalian sources.

TGF beta1 was originally identified as a mitogen (Roberts *et al.* 1981; Moses *et al.* 1981), but has since proven to have growth inhibitory effects on many cell types in culture (Moses *et al.* 1985). It also has tissue-specific effects on cellular differentiation *in vitro*, inducing differentiation of some cell types (Masui *et al.* 1986; Seyedin *et al.* 1986), whilst inhibiting differentiation of others (Massague *et al.* 1986; Ignotz and Massague, 1985). One major biological activity of TGF beta1 is its ability to induce deposition of extracellular matrix (ECM) by mesenchymal cells. This is mediated by increased biosynthesis of ECM components (Roberts *et al.* 1986; Ignotz and Massague, 1986) and of protease inhibitors and by decreased synthesis of extracellular proteases (Lund *et al.* 1987; Edwards *et al.* 1987).

TGF beta2 protein was isolated from demineralised bovine bone on the basis of its cartilage-inducing activity in culture (Seyedin *et al.* 1987). It was later found to be structurally and functionally related to TGF beta1 (Seyedin *et al.* 1987)). The spectrum of *in vitro* biological activities of TGF beta1 and TGF beta2 are very similar, though there are some marked specificities as well. TGF beta2 has potent mesoderm-inducing activity in a *Xenopus* bioassay (Rosa *et al.* 1988), whereas TGF beta1 can only act in a synergistic capacity along with fibroblast growth factor (FGF) in this assay (Kimelman and Kirschner, 1987). Conversely, of the two, TGF beta1 is the most potent growth inhibitor of endothelial cells in culture (Jennings *et al.* 1988). In the mouse embryo, TGF beta2 RNA has been shown to have a very different spatial and temporal expression pattern from that of TGF beta1 RNA, being localised to the mesenchymal components of tissues such as bone, cartilage, blood vessels and gut (Pelton *et al.* 1989). This gives further support to the proposition that these two very similar molecules serve different *in vivo* biological functions.

TGF beta3 was isolated by molecular cloning of cDNAs (ten-Dijke *et al.* 1988; Jakowlew *et al.* 1988*b*; Derynck *et al.* 1988), and there is comparatively little data available on its biological activity and role in embryogenesis.

Materials and methods

Mouse stocks

Mouse embryos were obtained from Parkes females mated with NIH males. The day on which the copulation plug was found was called day 0.5. All tissues were fixed overnight in ice-cold 4 % paraformaldehyde in phosphate-buffered saline, then dehydrated and embedded in paraffin wax. The palates of embryos 14.0 days gestational age or older were dissected in ethanol to ascertain the developmental stage of palatogenesis prior to paraffin-embedding.

Probe synthesis

^{35}S-labelled riboprobes were generated to a specific activity of 10^9 disints per min μg^{-1} using either the T3 or T7 polymerase transcription systems. Probes were digested to an average of 100 nucleotides by controlled alkaline hydrolysis (Cox *et al.* 1984) and used at a final concentration of 30 pg μl^{-1} in hybridisations.

The TGF beta1-specific antisense probe was a 600 nucleotide *Apa*I–*Kpn*I fragment subcloned into Bluescribe (Stratagene) from the full-length murine TGF beta1 cDNA (Derynck *et al.* 1986) which was kindly provided by Dr R. Derynck (Genenteck). The subclone corresponds to the precursor region of the TGF beta1 polypeptide (amino acids 68–268).

The control probe used was a full-length TGF beta1 human cDNA, kindly supplied by Dr G. Bell (unpublished). It was also subcloned into the Bluescribe vector in sense orientation with respect to the T7 promoter.

The TGF beta2 DNA probe was obtained by amplification of the reverse transcriptase product of total mouse embryo RNA using the polymerase chain reaction (Saiki *et al.* 1988). The oligonucleotide primers spanned the initiation and termination codons. This probe was identical in nucleotide sequence to that reported by Miller *et al.* (1989*b*) (F. Denhez unpublished). This study used the full-length probe; however, a TGF beta2 specific, 501 nucleotide *Pst*I–*Sac*I fragment has shown identical hybridisation pattern (data not shown).

The TGF beta3-specific probe was a 732 nucleotide fragment spanning amino acid residues 8 to 251 of the precursor polypeptide (Denhez *et al.* 1990).

Nucleotide sequence homologies between the three gene-specific probes were 42 % (beta1 to beta2), 47 % (beta2 to beta3) and 36 % (beta1 to beta3) (Pelton *et al.* 1989; Denhez *et al.* 1990).

In situ *hybridisations*

In situ hybridisation to 7 μm tissue sections was performed according to the protocol of Wilkinson *et al.* (1987) using 60 % formamide in the hybridisation mixture and a hybridisation temperature of 52 °C. The slides were dipped in Ilford K5 emulsion and exposed for 3, 7 and 21 days. After development the slides were stained in haematoxylin and mounted. Photomicrography was performed on an Olympus BK2 microscope using Panatomic X film (Kodak).

Results

The differential distributions of RNAs encoding the three related growth factors, TGF beta1, beta2 and beta3 were investigated during murine palatogenesis from 11.5 to 15.5 days gestational age. *In situ* hybridisation was performed on 7 μm coronal sections of the embryonic head using radioactive gene-specific probes complementary to each transcript. As a negative control, a human TGF beta1 sense probe was employed which gave no specific hybridisation signal (data not shown).

No specific hybridisation of any probe was seen in the very early palatal shelves at 11.5 to 12.5 days gestational age (data not shown). The failure to localise transcripts at this stage may relate to the limits of detection of the

in situ hybridisation technique. The first appearance of TGF beta transcripts occurred at the late vertical shelf stage. Two patterns of RNA distribution were seen at this time and later in shelf development. TGF beta1 and beta3 are expressed in the medial edge epithelia, whereas TGF beta2 RNA is localised in the underlying mesenchyme.

Late vertical palatal shelves

At 13.5 days gestation the TGF beta1-specific probe showed strong hybridisation to the submandibular gland (Fig. 1C), as previously shown by Lehnert and Akhurst (1988). At this stage, the palatal shelves, which are growing vertically, show no specific hybridisation to this probe.

The first TGF beta gene to show high level expression in the palatal processes is that for TGF beta3 (Fig. 1G,H). This gene is expressed in the epithelial component of the palatal shelves in the region that will give rise to the future medial edge epithelium. In several embryos, examined the spatial extent of the hybridisation signal is identical. TGF beta3 expression is more extensive in the vertical epithelium facing the tongue and stops abruptly on the oral side. In the most anterior region of the oro-nasal cavity, the epithelium of the nasal septum, which is also destined to fuse with the palatal shelves, shows high level TGF beta3 expression (Fig. 1G).

There is a low level of hybridisation of the TGF beta3 probe to the mesenchyme of the tongue, mandible and the upper regions of the maxillary processes. The mesenchyme immediately adjacent to the medial edge epithelium is, however, markedly devoid of autoradiographic signal. This observation was also made at later stages (see Figs 2F,G and 3D).

At this early stage, TGF beta2 does not appear to be specifically expressed in the palatal processes, although a characteristic signal is seen in the differentiating olfactory epithelium of the nasal process (data not shown). On some sections, small regions of the medial edge epithelia appear to show hyperplasia, resulting in small blebs of stratified epithelium (Fig. 1E). The epithelial cells within these hyperplastic nodules express very high levels of TGF beta2 RNA (Fig. 1D,F), whereas there is no hybridisation with either TGF beta1 or beta3 (data not shown).

The horizontal palatal shelf

After elevation of the palatal shelves at around 14.0 days gestation, the medial edge epithelia come into almost immediate contact (Fig. 2A). At this stage, TGF beta1 is now detectable in the medial edge epithelia in the same cells that express TGF beta3 (Fig. 2B,C,D). The expression of epithelial TGF beta1 RNA is correlated with the presence of the polypeptide in the underlying mesenchyme (Heine *et al.* 1987). TGF beta1 expression is also seen in regions of membranous ossification within the developing maxilla (Fig. 2B), as previously reported by Lehnert and Akhurst (1988).

By this time the quantity of TGF beta3 RNA in the medial edge epithelium has increased considerably. There is striking hybridisation with this probe in both in the medial edge epithelia and the epithelium of the oral aspect of the prefusion anterior nasal septum (Fig. 2F,G,H). In fact, the palatal epithelium at this stage is by far the richest source of TGF beta3 RNA within the entire embryo (F. Millan and R. Akhurst unpublished).

TGF beta2 RNA is excluded from the palatal epithelium, but a gradient of expression is seen within the mesenchyme beneath the medial epithelium, with highest expression levels immediately adjacent to the epithelium (Fig. 2E).

The fusing palate

With fusion of the medial edge epithelia to form the epithelial seam, the expression patterns established in the horizontal shelves continue. TGF beta1 (Fig. 3B) and TGF beta3 (Fig. 3D,G,H) RNAs are localised to the epithelial cells of the seam. This expression is lost as the seam disrupts and the cells lose their epithelial phenotype by transformation into mesenchymal cells.

TGF beta2 transcript levels increase and are now easily seen in the mesenchyme on either side of the seam (Fig. 3C,E,F). The RNA distribution is asymmet-

Fig. 1. Localisation of TGF beta1, beta2 and beta3 in vertical palatal shelves. Coronal sections are presented from 13.5-day embryos through the anterior third of the vertical palatal shelves. A and E,F,G,H are bright-field images, B,C,D are dark-field. (A) Bright-field image showing tongue (to), palatal shelves (ps) and nasal septum (ns). (B) Dark-field images showing non-specific hybridisation with sense probe. (C) TGF beta1 probe showing hybridisation to the submandibular gland (sm). (D) Non-specific mesenchymal hybridisation of TGF beta2 probe with localised area of expression in the epithelium of the left palatal shelf. (E) High-power image of adjacent section to D showing localised hyperplasia (hy) in the epithelia of one palatal shelf (ps). (F) High-power view of epithelial expression in D. (G) Hybridisation of the TGF beta3 probe to the medial epithelia of the palatal shelves and the oral epithelium of the nasal septum. (I) High-power view of the palatal shelf (ps) epithelial expression (boxed area) in H. Scale bar (A,B,C,D,G) represents 200 micrometers, scale bar (E,F,H) 50 micrometers.

Fig. 2. TGF beta1, beta2 and beta3 expression in the horizontal palatal shelves. All sections are from 14.0-day embryos. (A) Coronal bright-field image of horizontal, middle third palatal shelve (ps) medial edge epithelia (me) with the nasal septum (ns) above and oral cavity (oc) below. (B) Adjacent section to A with TGF beta1 probe hybridising to the maxilla (ma) and medial edge epithelia (me). (C) Bright-field paramedial sagittal section through posterior medial edge epithelia (me). (D) Dark-field image of C with TGF beta1 probe specifically hybridising to the medial edge epithelium. (E) Dark-field image of coronal section TGF beta2 hybridising to the medial and nasal palatal mesenchyme. (F) TGF beta3 hybridising to the medial edge epithelia on adjacent section to A. (G) Dark-field image of coronal section through middle third of palate, posterior to F, TGF beta3 probe hybridises to the medial edge epithelia. (H) High-power view of the palatal shelves (ps) and medial edge epithelia (me) expression in G (boxed area). Scale bar (A,B,C,D,E,F,G) represents 200 μm and in (H) 50 μm.

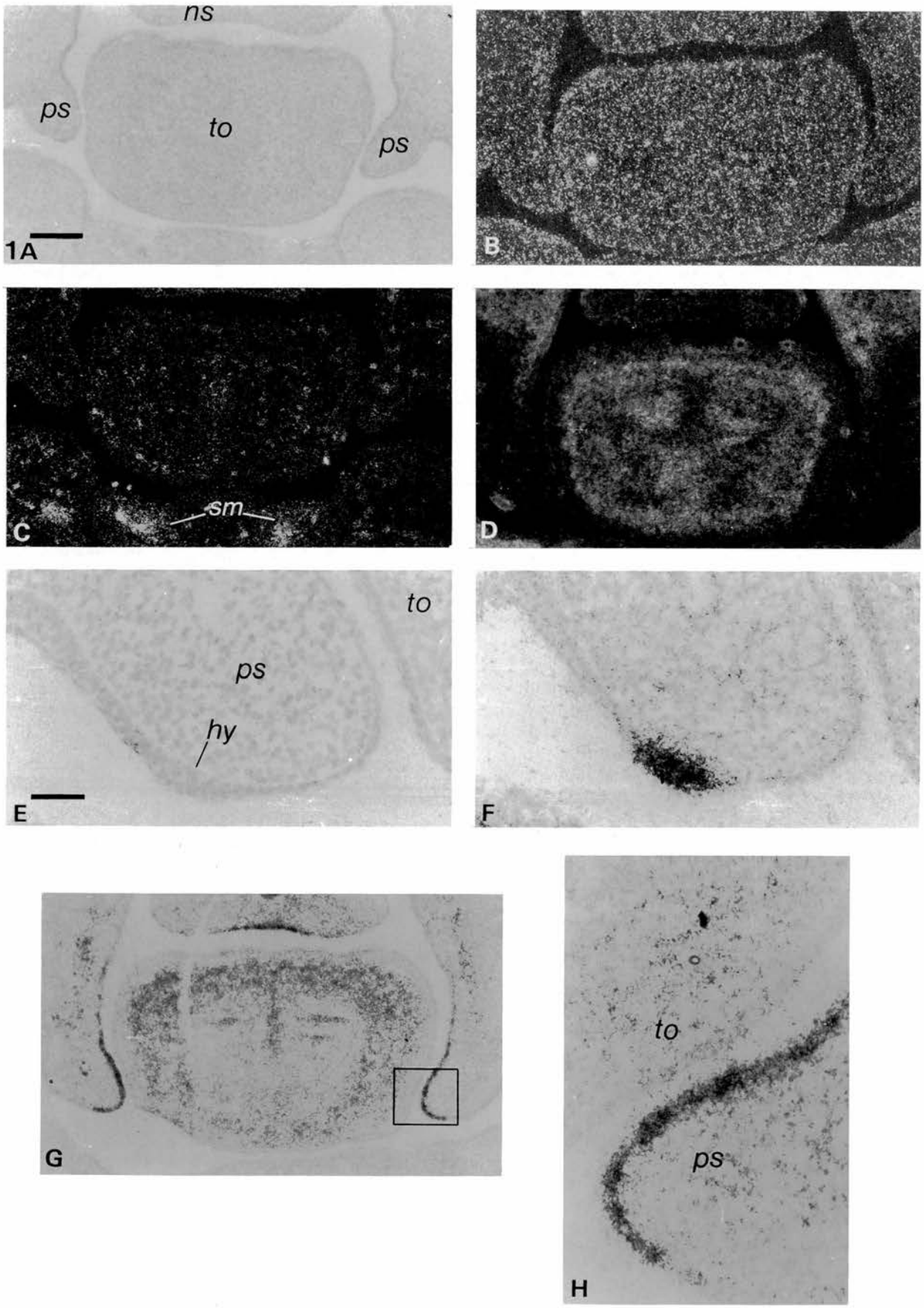
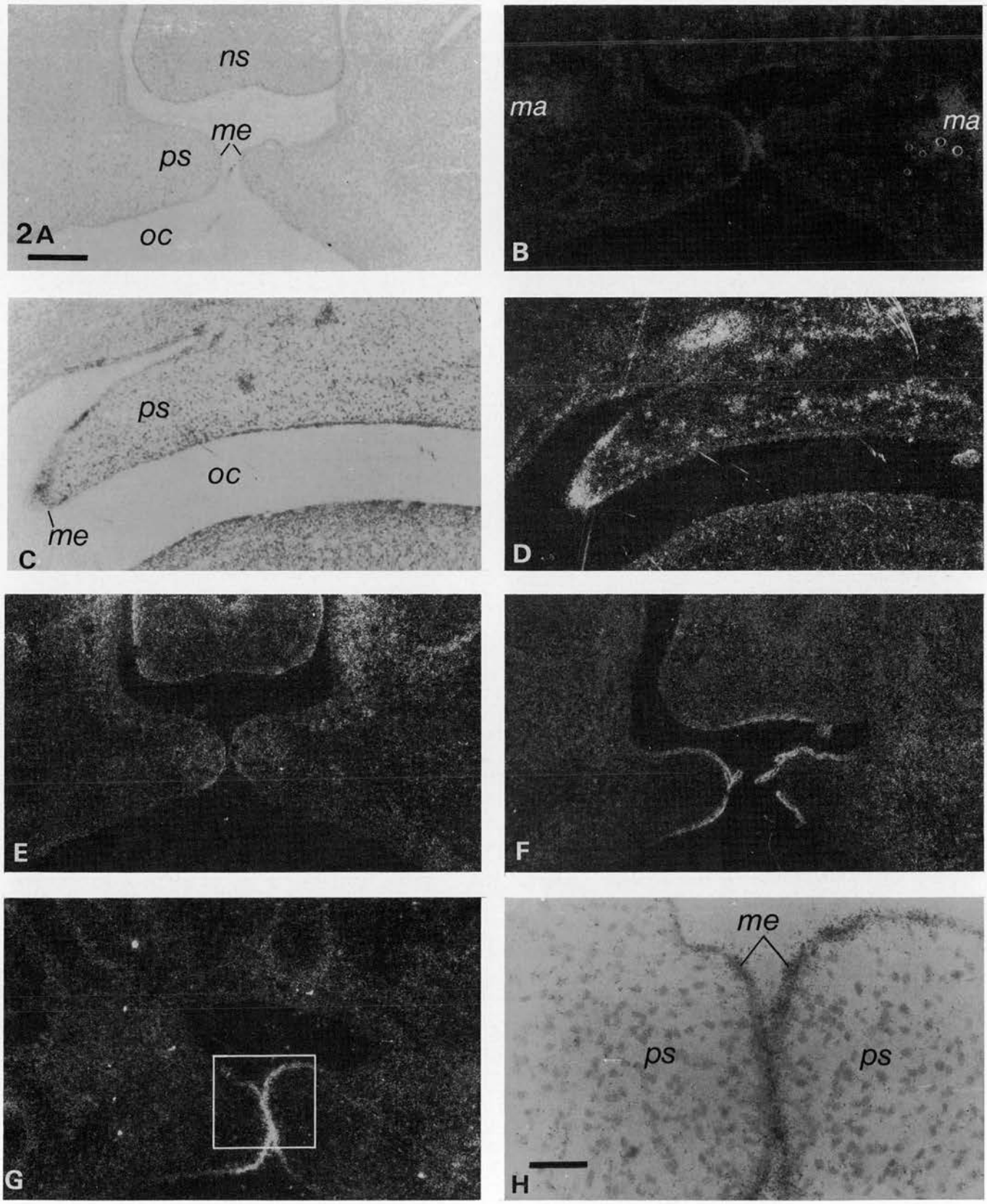


Fig. 1. For legend see p. 587



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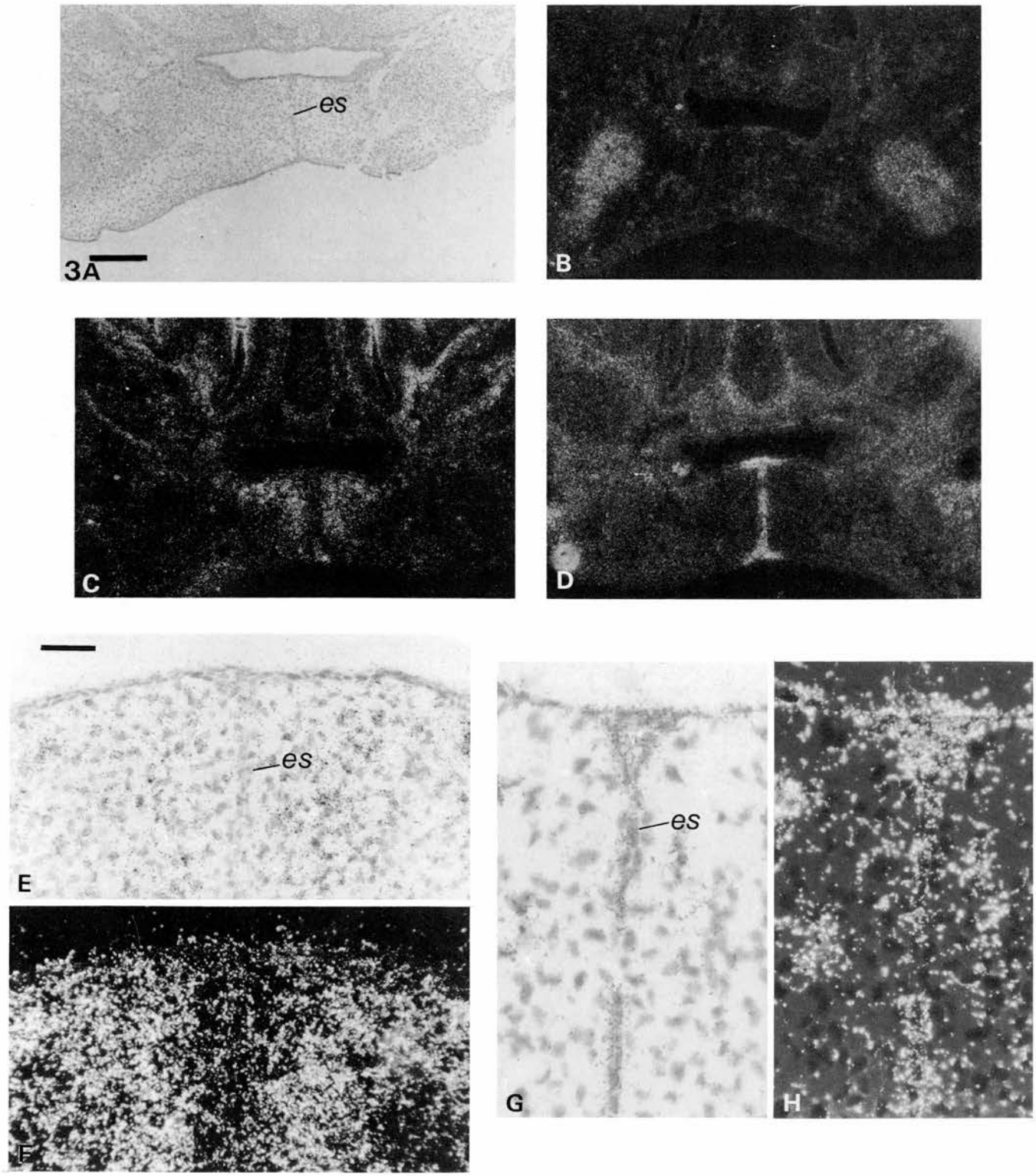

Fig. 3. Localisation of TGF beta1, beta2 and beta3 in the fusing palate. All sections are coronal through the middle third of the fusing palate of 14.5-day embryos. (A) Bright-field image of fusing palate with the midline epithelial seam (es). (B) Dark-field image showing TGF beta1 faint hybridisation to the epithelial seam. (C) Dark-field image of TGF beta2 hybridisation to the medial and nasal palatal mesenchyme. (D) Dark-field image of specific hybridisation of TGF beta3 to the epithelial seam. (E) High-power bright field image of the epithelial seam (es) in C showing absence of TGF beta2 expression in the seam. (F) Dark-field image of E. (G) High-power bright field image of TGF beta3 expression in the disrupting epithelial seam (es), showing hybridisation only where the seam is intact. (H) Dark-field image of G. Scale bar (A,B,C,D) represents 200 μm and (E,F,G,H) 50 μm.

ric with a higher concentration towards the nasal side of the palate.

The maturing palate

After fusion, the midline epithelial seam rapidly disrupts by a process of epithelial–mesenchymal transformation and cell death, thus establishing mesenchymal continuity (Fitchett and Hay, 1989). The fate of the palatal mesenchyme is regionally determined, anteriorly becoming ossified to form the bone of the hard palate and posteriorly giving rise to muscle (Ferguson, 1988).

TGF beta2 continues to be expressed in a diffuse region of the mesenchyme around the midline of the palate (Fig. 4C). Other studies from this laboratory suggest that this TGF beta2 expression may be associated with condensation of mesenchymal tissue prior to chondrification (D. Gatherer, F. Millan, D. Baird and R. Akhurst unpublished).

Postfusion, TGF beta3 expression also switches to become primarily mesenchymal. Hybridisation is seen in the perichondrium of the nasal septum as well as in the mesenchyme of the anterior secondary palate (Fig. 4D). In general, we have found that TGF beta3 expression is associated with chondrification in early mouse and human embryos (D. Gatherer, F. Millan, D. Baird and R. Akhurst unpublished).

Finally TGF beta1 expression in the maturing palate is limited to areas of ossification within the palate and around the nasal processes (Fig. 4B), as previously reported (Lehnert and Akhurst, 1988).

Discussion

Cleft palate is amongst the most common of congenital

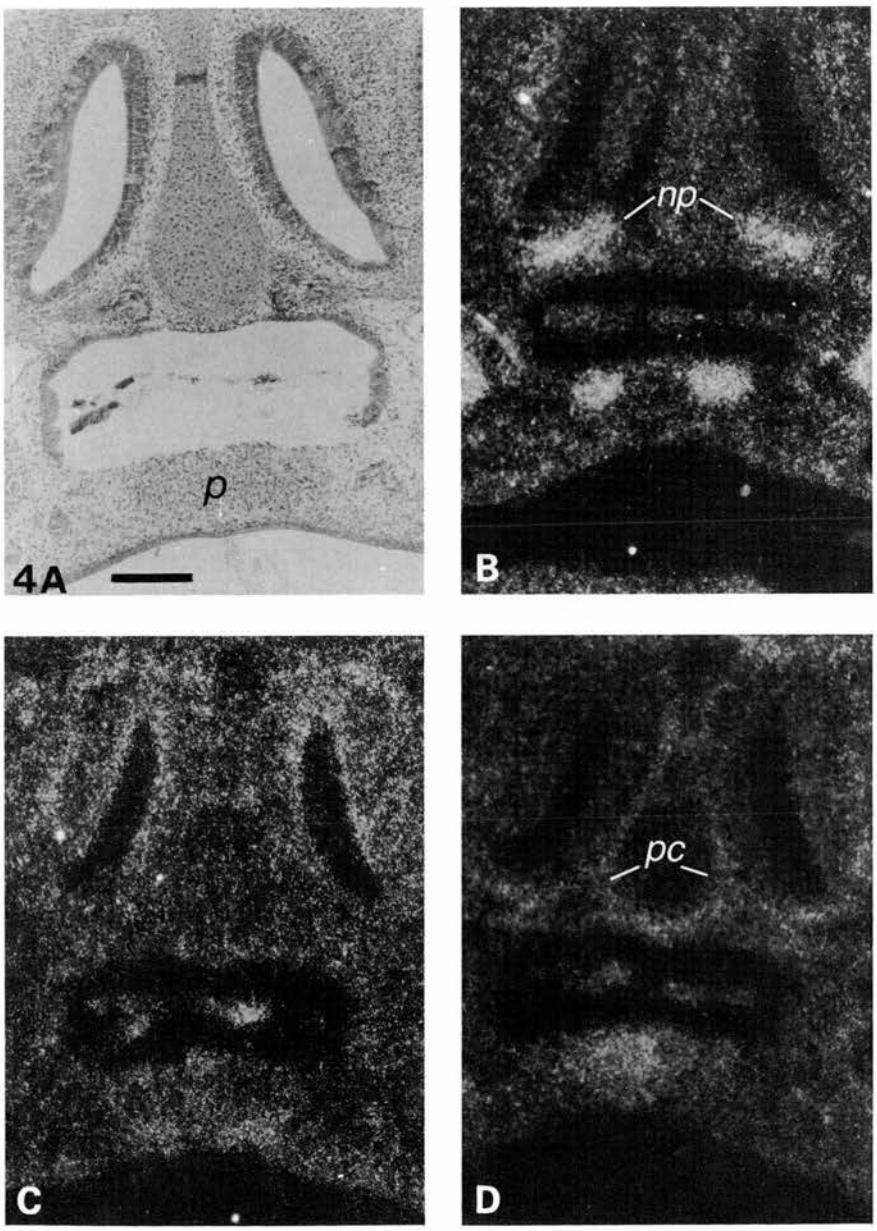

Fig. 4. TGF betas in the maturing palate. Sections have been cut coronally through the middle third of the palates of 15.5-day embryos. A is a bright-field image and B,C,D are dark-field. (A) The maturing palate (p). (B) TGF beta1 probe hybridising to the areas of ossification within the palate and around the nasal processes (np). (C) Diffuse hybridisation of the TGF beta2 probe to the mesenchyme around the midline of the palate. (D) Specific perichondrial (pc) hybridisation of the TGF beta3 probe around the nasal septum and the midline mesenchyma of the palate. Scale bar represents 200 μm.

malformations in humans (Thompson and Thompson, 1986). From a clinical standpoint, it would therefore be very important to understand the mechanisms controlling mammalian palatogenesis at the cellular and molecular level. The mouse represents a good model for such a study since palatogenesis is almost identical to that in the human, and the process can be disturbed in a controlled manner by the administration of teratogens (Morriss, 1973; Abbott *et al.* 1988). Furthermore, several genetic lines of mice are available which show increased susceptibility to this malformation (Fitch, 1957; Gasser *et al.* 1981).

The first evidence of the expression of members of the TGF beta family during palatogenesis is the epithelial expression of TGF beta3 in the vertical palatal shelves. TGF beta1 shows a similar pattern of expression to that of TGF beta3 but apparently with lower transcript prevalence. A comparison of relative transcript levels of different genes by *in situ* hybridisation is difficult to make, despite control over the size and specific activity of the radioactive probe. However, one conclusion that can be drawn regarding transcript prevalence is that the medial edge epithelial cells are the most abundant source of TGF beta3 RNA within the murine embryo at this period of development.

In addition to the epithelial expression of TGF beta1 and beta3, there is a distinct mesenchymal distribution of TGF beta2 gene expression. The temporal and spatial distribution of transcripts from these three genes is summarised in Fig. 5.

As yet there are no published reports of the polypeptide distributions of TGF beta2 or TGF beta3 during murine embryogenesis, though the mesenchymal distribution of TGF beta1 polypeptide in the palate (Heine *et al.* 1987) would be consistent with a paracrine mode of action of epithelially derived TGF beta1 on the underlying mesenchyme, as previously suggested (Lehnert and Akhurst, 1988; Akhurst *et al.* 1990*b*). From the similar pattern of epithelial TGF beta 3 gene expression one might speculate that this growth factor also has paracrine activities on the mesenchyme.

TGF beta1 (and beta3?) may act on the underlying mesenchyme in a variety of ways. TGF beta1 is known to stimulate proliferation and chemotaxis of cells of mesenchymal origin (Moses *et al.* 1985; Postlethwaite *et al.* 1987), though Sharpe and Ferguson (1988) have claimed that, in the palate, it is inhibitory to mesenchymal cell growth. Both cell migration and proliferation are essential features of palatal shelf development.

The action of TGF beta1 on the accumulation of ECM components has been the subject of extensive study. This growth factor induces synthesis of collagens and fibronectin (Roberts *et al.* 1986; Ignotz and Massague, 1986), tenascin (Pearson *et al.* 1988) and chondroitin/dermatan proteoglycans (Hiraki *et al.* 1988; Sharpe and Ferguson, 1988). Accumulation of the latter class of molecules is thought to be important in palatal shelf elevation by virtue of the rise in osmotic pressure resulting from hydration of the proteoglycan network (Pratt *et al.* 1973; Brinkley and Morris Wiman, 1987). In this respect it is interesting that high

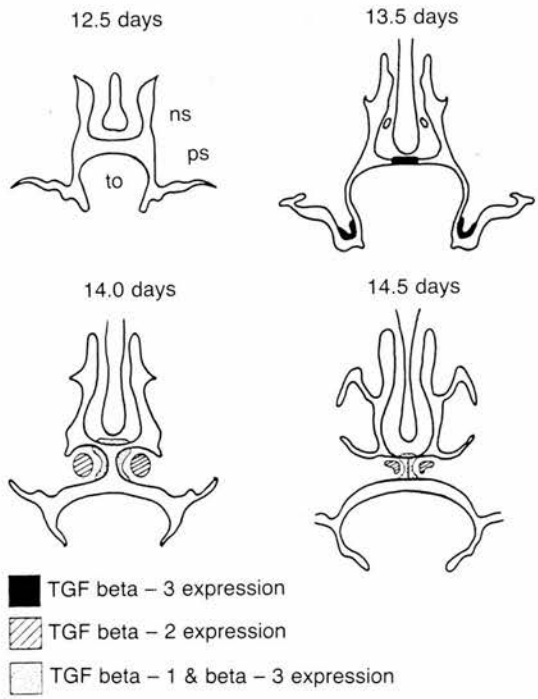

Fig. 5. Diagrammatic representation of the role of the TGF beta family in the developing palate. The line drawings represent coronal sections through the midpalate at the gestations (in days) indicated above the drawings. The diagrams show the palatal shelves (ps) growing down beside the tongue (to) and then elevating to fuse between the tongue and nasal septum (ns). The filled black areas illustrates TGF beta3 expression, diagonal striping for TGF beta2 and stippled areas for the combined expression of TGF beta1 and beta3.

levels of TGF beta3 RNA are observed 24 h prior to palatal shelf elevation.

The distributions of many extracellular matrix proteins are fairly ubiquitous within the palatal mesenchyme. Two significant exceptions are collagen IX and tenascin. Collagen IX appears on the cell surface of medial edge epithelial cells prior to shelf elevation (Ferguson, 1988). Tenascin is localised beneath the medial edge epithelium prior to and during palatal shelf fusion (Sharpe and Ferguson, 1988). Since TGF beta1 is known to induce synthesis of both of these proteins (Sharpe and Ferguson, 1988; Pearson *et al.* 1988), it is a reasonable supposition that these ECM molecules may mediate some of the effects of TGF betas. The distribution of tenascin is particularly significant since the embryonic distribution of this molecule is almost completely correlated with the presence of epithelial TGF beta1 RNA (Chiquet-Ehrismann *et al.* 1986; Lehnert and Akhurst, 1988; Akhurst *et al.* 1990*a*; Sharpe and Ferguson, 1988).

Tenascin can disrupt epithelial sheet continuity by breaking cell–cell and cell–substratum contacts (Chiquet-Ehrismann *et al.* 1989). It also specifically promotes the mobility of neural crest cells *in vitro* (Halfter *et al.* 1989). Both of these events would be necessary for

fusion along the mid-line seam, a time when the epithelial sheet disrupts, epithelial cells transdifferentiate to a mesenchymal cell phenotype and there is much cell mixing.

Terminal differentiation, or 'programmed cell death', plays an equally important role to transdifferentiation in the disruption of the midline epithelial seam (Pratt and Martin, 1975; Greene and Pratt, 1976). Cessation of epithelial DNA synthesis occurs 24 h prior to fusion (Pratt and Martin, 1975). It is accompanied by a down-regulation in epidermal growth factor (EGF) receptors (Abbott *et al.* 1988), and is not dependent on shelf contact *in vitro*. This lethal differentiation is specific to the medial edge epithelium, it is not seen in the oral or nasal components (Pratt and Martin, 1975; Tyler and Koch, 1975). The activation of TGF beta3 gene expression occurs 24 to 36 h prior to fusion and could be important in this growth inhibitory process.

TGFs beta1, beta2 and beta3 are each known to be growth inhibitory to epithelial cells, antagonising the mitogenic activities of TGF alpha and epidermal growth factor (EGF) (Massague, 1985; Like and Massague, 1986; Coffey *et al.* 1988; Grayar *et al.* 1989). It is, however, unlikely that EGF receptor down-regulation (Abbott *et al.* 1988) is directly initiated by TGF betas. Although this is a mechanism of negative growth regulation utilised by endothelial cells (Takehara *et al.* 1987), TGF beta acts distally to the EGF receptor in growth inhibition of all epithelial cells that have been examined (Massague, 1985; Like and Massague, 1986; Coffey *et al.* 1988).

TGF beta2 RNA distribution during palatogenesis is in marked contrast to that of TGFs beta1 and beta3. Its predominant localisation in the mesenchyme would agree with the observations of Pelton *et al.* (1989). It was suggested by Pelton *et al.* that mesenchymal expression of TGF beta2 might be important, not only in modulating the mesenchyme *per se*, but in supporting growth of the overlying epithelium *via* secondary events such as induction of TGF alpha. In this context, it is interesting that the TGF beta2 RNA distribution is asymmetric with respect to the nasal and oral regions. Differential concentrations of growth factors within the mesenchyme could contribute to the generation of regional heterogeneity of the overlying epithelium.

The localised high level expression of TGF beta2 in the hyperplastic nodules of the early medial edge epithelium is consistent with this growth factor acting as an inducible homeostatic regulator of epithelial growth and differentiation. Pelton *et al.* (1989) previously observed the expression of this growth factor in the suprabasal keratinocytes of the embryonic skin at a time when the rate of keratinocyte cell division would be slowing.

One point raised by these RNA localisation studies is the question of why genes encoding proteins with such similar *in vitro* biological activities should be expressed in the same developing organ, in some cases with overlapping but distinct patterns of expression, and in other cases with quite disparate transcript localisations. One explanation of the apparent temporal sequence of expression (TGF beta3 followed by TGF beta1 and beta2) would be the induction of one member of the TGF beta family by another, to amplify specific prefusion biological effects. It is known that TGF beta1 positively regulates its own expression in normal and transformed cells (Van Obberghen-Schilling *et al.* 1988) but little is known about 'cross-talk' between these individual genes. Clearly these different genes must serve some disparate *in vivo* biological functions. This is supported by the fact that some *in vitro* biological activities reported for the TGF betas do show some specificity in isoform requirement (Rosa *et al.* 1988; Jennings *et al.* 1988).

We conclude from this study that all three TGF beta isoforms must play an important role in mammalian palatogenesis. It will be interesting to see how expression of these genes is modified following treatment with teratogens that cause cleft palate, and in mutant mice with increased susceptibility to this deformity.

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TRANSFORMATING GROWTH FACTOR BETAS IN MAMMALIAN EMBRYOGENESIS

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Type β transformating growth factors (TGFβs) are members of a large superfamily of related proteins, each of which plays a pivotal role in embryonic processes. The TGFβs per se *are at least five in number, though only three isoforms have been identified in mammals. Here we will review the evidence, taken from* in vitro *studies on bioactivity and histochemical localization of RNAs and encoded proteins* in vivo, *that TGFβ1, β2 and β3 are involved in several mammalian developmental processes, including control of growth, differentiation, tissue inductions and morphogenesis.*

Key words: Transforming growth factor β, embryogenesis, cardiogenesis, epithelial-mesenchymal interactions, morphogenesis, extracellular matrix.

INTRODUCTION

It has become widely accepted that growth factors are not only involved in the regulation of cellular mitogenesis, but also in the modulation of a variety of biological processes at the cellular and tissue organizational levels. The transforming growth factor βs (TGFβ) are an excellent example. These growth factors can influence cell growth, differentiation, inductive interactions, transdifferentiation and cell lineage pathways, as well as modulate the biosynthesis of the extracellular matrix, all of which are of central importance in embryogenesis.

The *in vitro* biological activities of TGFβ are variable, depending on cell type, culture conditions and the presence of other growth factors. These have recently been

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extensively reviewed elsewhere [1–3], and thus will only be mentioned in the context of their relevance to specific development processes.

THE TGFβ SUPERFAMILY

TGFβ1 was identified and isolated around a decade ago, on the basis of its ability to induce anchorage-independent growth of non-transformed cells [4–6]. The mature growth factor was shown to be a 25 kDa homodimeric protein. Once the gene for TGFβ1 was cloned [7], it became clear that many molecules, from a diverse range of species, were related to TGFβ1. These include the *Decapentaplegic (dpp)* gene of *Drosophila*, which is involved in dorso-ventral patterning of the fly [8] and the amphibian mesoderm-inducing factors, Vg1 and MIF-XTC (or activin B) [9,10] and also the mammalian proteins; Mullerian inhibitory substance, which aids the regression of male Mullerian duct during development [11], the inhibins and activins [12,13], and the bone morphogenetic proteins (BMPs) [14], which include Vgr-1 [15]. A striking observation is that each of these proteins serves a fundamental function(s) in embryonic processes, which led researchers to investigate a potential role for TGFβ *per se* in development of the embryo.

It is beyond the scope of this article to review the role of each of the superfamily members in embryonic development. In the interest of brevity we will therefore restrict ourselves to the function of the TGFβs *per se*, and focus on their role in *mammalian* development.

THE TGFβ FAMILY

All TGFβs are translated as large precursor proteins which are subsequently processed by the removal of an *N*-terminal signal sequence and cleavage of the larger precursor domain (latency-associated peptide, LAP) from the adjacent *C*-terminal active polypeptide. A dimer of LAP remains associated with the bioactive TGFβ homodimer, thus keeping it in a biologically latent form. Although factors capable of activating TGFβ *in vitro* have been identified, it remains unknown how TGFβ activation occurs *in vivo* (see [2]).

To date, five members of the TGFβ family have been reported. These are characterized by sharing 70% amino acid identity within the bioactive *C*-terminal portion of the polypeptide (in contrast, the superfamily members are only 30–40% homologous). TGFβ1, β2 and β3 have been isolated from various mammalian and avian sources, but, as yet, no mammalian homologues of TGFβ4 or β5 have been found. The latter two have only been isolated from chick and frog, respectively [16,17]. In contrast to the intraspecies divergence of family members, the interspecies amino acid sequence conservation of any individual TGFβ gene is virtually complete. This has raised the discussion of whether multiple TGFβs have arisen to serve distinct biological functions *in vivo*, or whether the purpose of the gene family is to provide expression of proteins of identical function in diverse tissue contexts.

Comparative biological activity studies *in vitro* suggest that TGFβ1, β2 and β3 have similar qualitative effects on keratinocytes, fibroblasts, osteoblasts and endothelial cells, but that these activities vary quantitatively [18–20]. In yet other biological

systems, the TGFβs clearly have distinct roles. As an example, mammalian TGFβ2 can induce the formation of mesoderm from *Xenopus* ectodermal explants. In contrast, TGFβ1 does not possess this bioactivity, but can synergize with fibroblast growth factor (FGF) in this process [21]. It thus appears more likely that the TGFβs do indeed possess functional specificity *in vivo*.

Since the bioactive coding portions of TGFβs are so highly conserved, it is thought that biological specificity may be conferred by the precursor region, either in differential targeting of the latent complex to its site of action, binding to cell surface receptors or activation of the latent forms. The precursor domains exhibit conservation of certain structural moieties, but also possess distinct intermolecular differences. TGFβ2, for example, unlike other isoforms, lacks a potential integrin-binding site [2], whereas TGFβ4 lacks a signal peptide [16].

STUDIES ON MAMMALIAN EMBRYOS

As yet there is no definitive evidence that any of the TGFβs are endogenous regulators of mammalian embryonic processes. Most of the information implicating TGFβs as embryonic modulators comes from descriptive studies on a) the ability of early embryos and embryonal stem (ES) cells to secrete bioactive TGFβs, b) immunohistochemical localization of TGFβs in mouse sections, c) detection of TGFβ RNAs by Northern blot or polymerase chain reaction (PCR) of total RNA, or by *in situ* localization in embryo sections. From this data, together with the extensive knowledge of the *in vitro* bioactivities of TGFβs gained from cell or organ cultures, various inferences may be made. Some elegant *in vivo* studies have also implicated TGFβ as important in morphogenesis of the mammary gland, but, as yet, it has not been demonstrated that any of the TGFβs occur endogenously within this developing organ [22].

Most of the published work relevant to the functions of TGFβ in embryonic processes has been gained from immunohistochemical protein localization studies [23,24] and *in situ* RNA localization studies [25–31]. However, conclusions drawn from these studies must be made with caution for a number of reasons. Firstly, the similarity of TGFβ isoforms at both nucleic acid and polypeptide levels has made the preparation of isoform-specific reagents exceedingly difficult. This is particularly true for the generation of antibodies. Cross-reactivity may not occur in one assay, but be extensive in another. Furthermore, antibodies raised against identical peptides can recognize different epitopes, possibly as a result of conformational changes in the protein following its translation, secretion and activation [32,33].

The two most extensively utilized polyclonal antibodies against TGFβ1 are the so called LC and CC reagents. These were raised against the same 30 amino acid peptide, yet show different staining patterns in immunolocalization studies, the former recognizing intracellular TGFβ1 and the latter staining extracellularly [32–34]. It has been postulated that these antibodies recognize the precursor and mature forms of TGFβ1, since tissues staining with the LC antibody are normally identical to, or in close proximity to those staining with the CC antibody [33,34]. Discrepancies do, however, occur, for example in the adult heart, intense LC staining is observed in the myocardium whereas CC staining occurs only in the valve leaflets [34].

The design of gene probes for *in situ* hybridization studies is less of a problem, but a

judicious choice of probes is warranted, since cross-reactivity can occur [26,31]. In general, the results of TGFβ1 RNA localization studies are in agreement with the immunolocalization of protein. Nevertheless, there are inconsistencies. The spleen, for example, is know to be the richest source of TGFβ1 RNA in the adult [35,36], nevertheless, no protein can be detected with the LC or the CC antibodies [34]. Also in the heart, the intensity of LC staining of the myocardium does not correlate with RNA levels determined by Northern blot or *in situ* hybridization [34,35,37]. These may represent examples of extreme post-transcriptional control of protein production. Finally, in the embryonic mouse lung, staining for pro-TGFβ1 is observed in the early bronchial epithelium [24], whereas no TGFβ1 RNA is detectable by *in situ* hybridization [26,31]. TGFβ3 RNA is, however, detected in the early pulmonary epithelium [31], opening up the possibility that the pro-TGFβ1 antibody also detects pro-TGFβ3.

Another limitation of descriptive studies is the inability to determine what percentage RNA is translated and what portion of the immunohistochemically detectable protein is biologically active and available to the responding cell. Although the half-life of TGFβ in serum is exceedingly short ($t_{1/2}$ = 2.5min), TGFβs can bind to the extracellular matrix, which could either act as a pool of the latent growth factor, readiness for future use, or as a sink for spent protein.

Finally, the presence of the growth factor does not necessarily imply that cells in its vicinity can respond. Although it has been widely reported that TGFβ receptors are ubiquitous on normal cells [38], alterations in ligand-binding have been observed during differentiation of cells in culture [39]. A clear example of the differential responsiveness of similar cell types to TGFβ *in vivo* comes from work on the mammary gland. In this system, TGFβ-impregnated plastic implants potently inhibit DNA synthesis in pubertal ductal epithelial cells of the growing end buds, but have no effect on 'maintainence' DNA synthesis in lumenal epithelial cells nor on the rapidly growing secretory alveolar epithelial cells of the pregnant animal [22].

TGFβs IN PRE- AND PERI-IMPLANTATION EMBRYOS

Much of the evidence implicating TGFs as important in pre-implantation development has come from the study of embryos *per se*, or of embryonal carcinoma (EC) or embryonal stem (ES) cell lines, which are thought of as models of the pre-implantation embryo [39–42]. Several years ago it was shown that EC cells release, but do not respond to, TGFs, whereas their differentiated derivatives are TGF-responsive [40]. This was correlated with the novel appearance of high affinity receptors of TGFβ on the cells following induction of differentiation [39]. Extrapolating to the *in vivo* situation, it has been predicted that TGFβ might act in a paracrine manner, whereby the undifferentiated cells of the inner cell mass are the source of TGFβ which modulates growth and/or differential of the endodermal derivatives. Indeed, TGFβ1 is synthesized by pre-implantation embryos, though with no obvious regional distribution [42,43].

More recently it has been shown that TGFβ2 is also synthesized in pre-implantation embryos, though in this case endodermal outgrowths of the blastocyst, rather than undifferentiated inner cell mass cells, are the source of growth factor [41].

The exact function of the TGFβs in peri-implantation development remains

debatable. They could be involved in autocrine or paracrine control of cell growth/differentiation, elaboration of the extracellular matrices synthesized by the implanting embryo [41] or in interactions with maternal tissue, for example in inducing angiogenesis at the site of implantation [42].

HAEMATOPOIESIS

The first evidence of high concentrations of TGFβ1 RNA within the murine conceptus occurs within the extraembryonic blood islands at 7 days *post-coitum* (*p.c.*) [37]. At this early stage it is impossible to distinguish between haematopoietic and endothelial cell precursors, though we believe that both cell types express TGFβ1. The blood islands are the major sites of haematopoiesis in the early embryo. At later stages, haematopoietic stem cells migrate to the liver, and finally populate the adult spleen and bone marrow. In all of these organs, the haematopoietic progenitors are marked by high levels of expression of TGFβ1 RNA. This is particularly pronounced in proerythroid progenitors and megakaryocytes [26,27,30]. Since TGFβ1 is known to be a potent inhibitor of haematopoiesis [44], it is likely that this growth factor acts as an autocrine negative regulator of cell growth. Its presence at a time of rapid cell division may be necessary for controlled proliferation and/or differentiation.

Neither TGFβ2 or β3 RNAs have been detected in haematopoietic tissue of mouse or man [30,31].

VASCULARIZATION AND ANGIOGENESIS

TGFβ elicits an angiogenic response *in vivo* when injected subcutaneously [45], though it is not known whether this is a direct response of the endothelial cells, or secondary to events initiated in other cell types, such as macrophages [46]. Clearly, *in situ* hybridization studies would suggest that TGFβ1 is not only an endogenous autocrine regulator of vascularization and angiogenesis, but that this growth factor may serve such a role prior to the appearance (and thus involvement) of other cell types. Expression of TGFβ1 RNA is seen in endothelial cells of the blood islands as the extraembryonic blood vessels are laid down, and in the cardiogenic plate of the embryo proper at 7.5 days *p.c.* [37]. This expression is detectable very soon after the appearance of the three definitive germ layers and before overt differentiation of cell types, implicating this growth factor as a very early modulator of vascularization.

During vascularization and angiogenesis, endothelial cells perform several functions, all of which may be modulated by TGFβ. These include cell proliferation, tube formation, protease production and extracellular matrix production. Studies of TGFβ1 bioactivity on endothelial cells *in vitro* have been confused, since the cellular response depends on the precise culture conditions. In general, it can be summarized that TGFβ1 inhibits endothelial cell proliferation in a 2 dimensional (2-d) but not in a 3-d culture system, suggesting that TGFβ1 is an endogenous autocrine negative regulator of endothelial cell growth. In addition, in the 3-d culture system TGFβ1 promotes capillary-like tube formation [47,48].

The endothelial cell response to TGFβ is clearly isoform-specific *in vitro*. Though TGFβ1 is a potent growth inhibitor of this cell type, at physiological concentrations, TGFβ2 shows no such activity [49]. In this respect, it is notable that, of the three TGFβ

isoforms, *only* TGFβ1 RNA is detectable in endothelial cells [30,31]. This may give support to the theory that differential expression patterns within the embryo are related to functional specificity *in vitro*.

CARDIAC DEVELOPMENT

Progenitor cells of the mammalian heart appear in the splanchnopleuric mesoderm very early in embryogenesis, immediately following gastrulation. These cells become organized into two primitive tissue types, the endocardial tube which is encapsulated by the myocardial mantle. Initially, the endothelial and myocardial layers are separated by an extensive acellular basement membrane, the cardiac jelly. However, there is an early regional differentiation of the endothelium, such that some cells overlying areas which will give rise to the future septae and valves undergo a phenotypic transformation and infiltrate the cardiac jelly to form mesenchymal cushion tissue.

The mechanism of endothelial–mesenchymal transformation in chicks has been extensively studied by Markwald and his collaborators using an *in vitro* culture system [50–52]. It has been shown that a signal, arising from the myocardium, induces the overlying endothelium to undergo the transformation event. The inducing signal is produced in a regional manner ie. only from the myocardium of the atrioventricular (av) junction region and outflow tract. Furthermore, only the endothelia of the av junction and outflow tract, but not the atrial or ventricular endothelia, are competent to respond to this signal. Various lines of evidence suggest that TGFβs may be central to the events leading to the formation of mesenchymal cushion tissue and subsequent cardiac morphogenesis.

Potts and Runyan [53] performed tissue recombination experiments with dissected embryonic chick hearts. They demonstrated that, although ventricular myocardium could not induce mesenchymal transformation of av endothelium, if TGFβ was added to the assay the transformation proceeded. Additionally, they showed that antibodies which block the activity of TGFβs could also prevent the *in vitro* transformation of av endothelial cells induced by av myocardium. Thus, it appears that one of the TGFβs was, at least partly, involved in this regional transformation event.

Immunolocalization and *in situ* localization of murine TGFβ proteins and RNAs has also shown that both TGFβ1 and β2 are localized within tissues which contribute to endocardial cushion tissue formation [23, 26, 31, 37] (summarized in Fig. 1). TGFβ1 RNA is expressed ubiquitously in endocardial cells at early stages. This expression subsequently becomes restricted to endocardial cells which overly cushion tissue, and this restricted pattern persists in the cardiac valve leaflets until 1 week post-natally [37].

TGFβ1 expression is not limited to the period of cushion tissue induction and is thus unlikely to be involved in the inducing event *per se*. Its expression appears to be correlated with morphogenetic movements of the endothelium and mesenchyme. Endothelially-synthesized TGFβ1 protein is mainly localized in the underlying cushion tissue [23, 37], and this is correlated with the localization of extracellular matrix proteins, such as tenascin [37]. the function of TGFβ1 may, therefore, be to regulate growth of the endothelium in the vicinity of the heart valves, and to modulate production of extracellular matrix in cardiac cushion tissue, thus favouring tissue movements essential to remodelling of the heart.

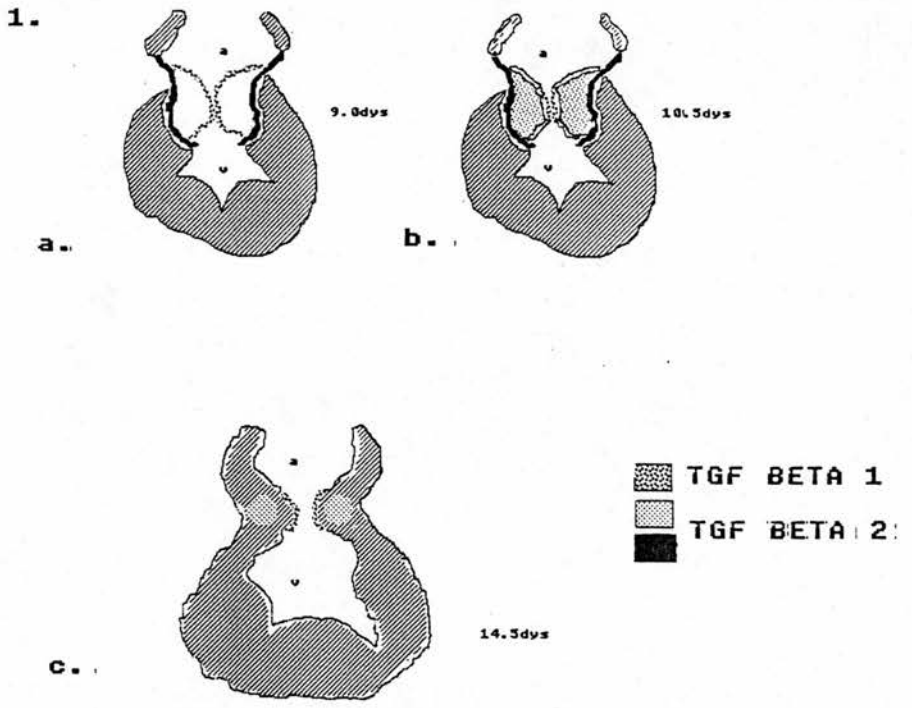


FIGURE 1. Schematic representation of the developing heart showing the pattern of expression of TGF β1 and β2 during atrioventricular valve (av) formation. As early as 9.5 days *p.c.*, before overt appearance of mesenchymal cushion tissue, there is a regionally restricted distribution of the TGF β2 transcripts within the myocardium underlying the av valve. TGFβ1 transcripts are localized to the endothelium (part a). The peak of cardiac TGFβ2 RNA occurs at 10.5 days *p.c.*, with a clear spatial restriction pattern to myocardium underlying mesenchymal cushion tissue (part b). By 14.5 days *p.c.* there is no expression of TGFβ2 in the myocardium surrounding the av valve, transcripts being restricted to the mesenchyme of the cushion tissue (part c). TGFβ1 RNA is still expressed in the endothelium at this stage and up to at least one week after birth. a = atria; v = ventricle.

In situ localization of murine TGFβ2 RNA, however, makes it a strong candidate for a component of the inducing signal. This gene is expressed transiently, and in a regionally-restricted manner, in the myocardium underlying the av junction and outflow tract at the time of cushion tissue formation. To a lesser extent, it is also expressed in the cushion tissue *per se* [31].

Since we have shown that av endothelium is a source of TGFβ1, it is surprising that there is a requirement for additional TGFβ in the bioassay used by Potts and Runyan [53]. This might be explained by species differences between mammals and avians, latency of the TGFβ1 protein, requirements for a certain threshold level of TGFβ or requirements for specific isoforms and/or cross-induction of the different TGFβs [2, 54].

SKELETAL DEVELOPMENT

TGFβ is known to have multiple biological effects on mesenchymal cells, chondroblasts, osteoblasts and osteoclasts, affecting cell proliferation, movement, differentiation, secretion of extracellular matrix molecules and even influencing developmental fate of osteoblast progenitor cells [55–57]. Furthermore, adult bone is one of the richest sources of this growth factor. It is, therefore, not surprising that all three TGFβ isoforms, plus the BMP family members [58], are expressed in areas of chrondrification and ossification. Expression of each isoform is, however, very specific, both with respect to the cell type, its state of differentiation and body site (see Table 1).

TABLE 1. Differential localization of RNAs encoding TGF β1, β2 and β3 during murine embryogenesis.

	β1[1]	β2[4,5]	β3[4,5]
Haematopoietic tissue	+	−	−
Endothelia	+	−	−
Thyroid	+	−	−
Parathyroid	+	−	−
Thymus	+	−	−
Epithelia			
Whisker follicles	+	+	+
Salivary gland	+	+	−
Tooth bud	+	+	−
Secondary palate[2]	+	−	+
Bronchial epithelium	−	+(s)	+(c)
Optic epithelium	−	+	−
Olfactory epithelium	−	+	−
Lens epithelium	−	+	−
Retina	−	+	−
Hyperplastic nodules	−	+[2]	−
Suprabasal keratinocytes	−	+[3]	−
Cartilage and bone			
Pre-cartilaginous blastema	−	+(limb)	+(iv)
Growth zone of long bone	−	+	−
Perichondria	−	−	+
Hypertrophic cartilage	−	−	−
Osteoblasts, osteoclasts	+	−	−
Cardiac tissue			
Pre-valvular endothelium	+	−	−
Pre-valvular myocardium	−	+	−
Neuronal tissue			
Ventral spinal cord	−	+	−
Ventral fore-brain	−	+	−
Muscle	−	(+)[6]	(+)[6]
Mesothelia	−	−	+
Mesenchyme	−	+	+

Notes; + denotes that RNA is abundant at some stage between 9.5 days *p.c* and birth, though expression may be transient. − is below the detection level of *in situ* hybridization.

[1] Data taken from [25–27]. [2] Data taken from [29]. [3] Data taken from [28]. [4,5] Data taken from [30,31]. [6] Low levels of hybridization are seen in some muscular tissues at some stages, but this is not abundant or ubiquitous.

c = cuboidal; s = squamous; iv = intervertebral disc anlagen.

TGFβ2 and β3 appear to be involved in early stages of formation of the skeletal system, including the condensation of mesenchymal cells to form cartilage precursors [30, 31]. For example, both of these genes are expressed in pre-cartilaginous blastemae; TGFβ2 RNA is seen in the limb buds, whereas TGFβ3 is expressed in the intervertebral disc anlagen [30, 31]. In this respect, it is interesting that both TGFβ2 and, to a lesser extent TGFβ3, are expressed at mesenchymal sites involved in cell proliferation, migration and condensation, such as facial and palatal mesenchyme [29–31]. TGFβ is known to stimulate proliferation and chemotaxis of mesenchymal cells in culture [59, 60] and, indeed, TGFβ1 and β2 were earlier isolated as factors that could induce the formation of chondroblasts from muscle from muscle mesenchymal cells in culture [61].

In contrast, TGFβ1 expression is associated with more overtly differentiated cell types in areas of ossification, namely osteoblasts, osteocytes and osteoclasts [25, 26, 30]. It has been reported that TGFβ2 is also expressed in these cell types [28], though this has been questioned by others [30, 31]. Unlike TGFβ2 and β3, TGFβ1 is, thus, more likely to be involved in control of osteoblast/osteoclast function, including bone remodelling which continues in the adult, and is influenced by osteotropic hormones [62].

TGFβS IN EPITHELIA AND IN EPITHELIAL–MESENCHYMAL INTERACTIONS

It has long been recognized that TGFβ1 is growth inhibitory to most epithelial cell types *in vitro* and *in vivo* [22, 63, 64]. This observation has now been extended to include TGFβ2 and β3, which are, in fact, more potent negative regulators of epithelial cell growth than TGFβ1 [18]. Additionally, some epithelial cell types are induced to differentiate by TGFβ [65–67]. It is, therefore, an interesting observation that all three TGFβ RNAs are expressed at high levels in various embryonic epithelia, often at times of rapid epithelial cell growth (see Table 1) [26, 28–31]. This has raised the question of whether epithelially-synthesized TGFβs are involved in regulation of growth and/or differentiation of the epithelium *per se*, or in paracrine modulation of the adjacent stroma.

Epithelial–Mesenchymal Interactions

TGFβ1 was the first of the isoforms reported to be synthesized in epithelial cells *in vivo* [68]. Surprisingly, the RNA is transiently detected, at high levels, in several specialized epithelia at times of most rapid cell division. Two circumstantial lines of evidence suggest, however, that the major function of the encoded protein is in paracrine interactions with the adjacent mesenchyme. Firstly, the RNA is only seen in epithelia that are actively involved in morphogenetic interactions with the adjacent mesenchyme, such as the salivary gland, tooth bud, whisker follicle and secondary palate [26, 29] (see Fig. 2). Secondly, in each of these situations the encoded protein is localized in the adjacent mesenchyme [23].

Bearing in mind the caveats with interpretation of *in situ* localization and immunolocalization data already discussed, bioactive TGFβ1 may act on the underlying mesenchyme in a variety of ways. It can induce proliferation, differentiation

2

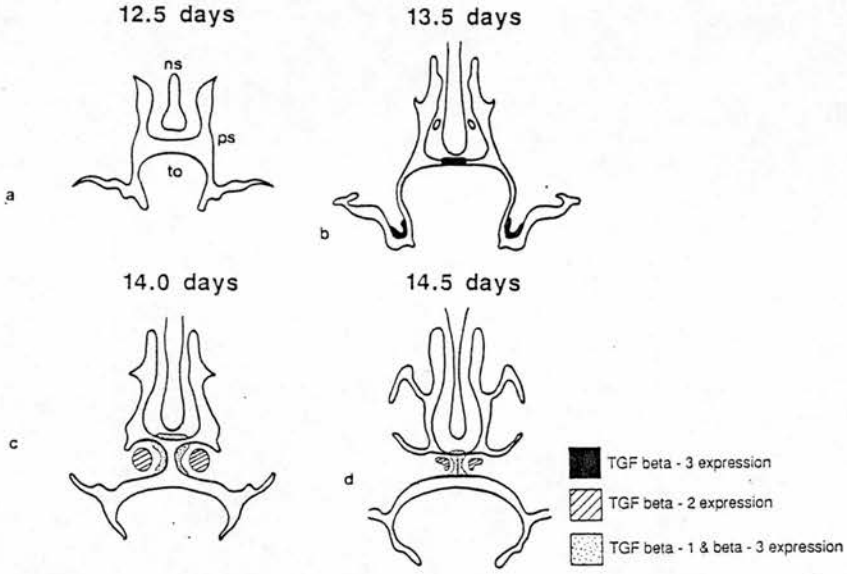


FIGURE 2. Diagrammatic representation of the role of the TGFβ family in palatogenesis. The line drawings represent coronal sections through the midpalate at the gestations (in days indicated above the drawings. The diagrams show the palatal shelves (ps) growing down beside the tongue (to) and then elevating to fuse between the tongue and nasal septum (ns). The filled black areas illustrate TGFβ3 expression, diagonal striping for TGFβ2 and stippled areas for the combined expression of TGFβ1 and β3.

and chemotaxis of mesenchymal cells [56, 59, 60], each of which occurs during morphogenesis. Secondly, it has a major effect on the elaboration of the extracellular matrix (ECM) and on the synthesis of cell surface receptors for ECM components (reviewed in [1]).

It is widely accepted that ECM composition can influence cell-cell and cell-substratum interactions, resulting in differences in cell migration or stabilization of organ structure. Studies in organ culture have emphasized the importance of ECM composition on branching morphogenesis of lung and salivary gland. It has been shown that collagen type III, for example, is essential to cleft formation in the developing salivary gland [69, 70].

Subsequent to studies on TGFβ1, it has been shown that, in each of the cases where this gene is activated in embryonic epithelia, the RNA co-localizes with that of TGFβ2 and/or β3 [29–31] (see Table 1). A good example is the transient epithelial expression of TGFβ1 and β3 during formation of the murine secondary palate [29] (Fig. 2.). Both of these genes are transcribed in the medial edge epithelium of the palatal shelves, as they form, elevate and fuse, and also in the epithelium of the anterior nasal septum, which is destined to fuse with the palate. No information exists on the localization of the corresponding TGFβ3 protein, but one might assume that the two isoforms serve similar paracrine functions within the palatal mesenchyme, stimulating cell proliferation and tissue migration [29].

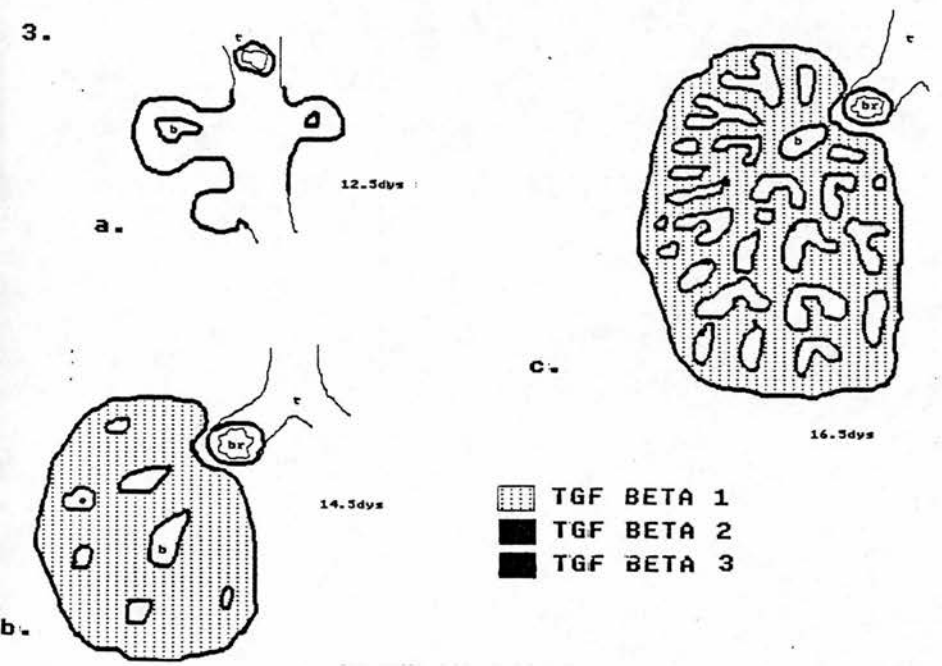
As in the aforementioned cases (Table 1), epithelially-synthesized TGFβ1 protein co-localizes in the mesenchyme with the ECM component, tenascin, which is one of the many ECM-encoding genes which is transcriptionally up-regulated by TGFβ[71]. This

bservation is particularly significant since tenascin is known to disrupt epithelial sheet ɔntinuity [72] and to promote the mobility of neural crest-derived cells [73]. Each of ıese bioactivities is obviously important in morphogenesis of the palate, salivary land, tooth and whisker follicle.

The Lung and Mammary Gland

The lung and mammary gland represent two further organs in which TGFβs might lay a central role in morphogenesis. Heine *et al.* [24], on finding co-localization of xtracellular TGFβ1 protein with collagens I and II III, fibronectin and proteoglycans ı the embryonic lung, have suggested that the ECM-directed branching of this organ ; controlled, in part, by epithelially-synthesized TGFβ1. Although TGFβs are most robably involved in lung morphogenesis, the cellular source of the growth factor(s) nd the isoform involvement may be debatable.

No expression of TGFβ1 RNA has been detected in bronchial epithelia, though ıidespread expression is seen in the stroma [26, 31]. In contrast, both TGFβ2 and GFβ3 RNAs are expressed in bronchial epithelia, albeit in different cell types (Fig. 3).



IGURE 3. Schematic representation of the role of TGF beta isoforms during lung development. THe drawings epresent sagittal sections through lungs from 12.5 days *p.c.* to 16.5 days *p.c.* TGFβ1 RNA is restricted to the ıesenchymal cells of the lung most notably at 14.5 days *p.c.* when the lung is not fully mature. TGFβ3 RNA is een submucosally in the trachea and proximal bronchi at 12.5 days *p.c.* Simultaneously, intense expression is een in the immature columnar epithelial cells of the growing bronchioles (a). As lung outgrowth proceeds, GFβ3 RNA expression remains restricted to the columnear epithelial cells of the proximal respiratory tree. No GFβ3 expression is seen in the simple cuboidal epithelia of the growing terminal end buds (b). TGFβ2 is estricted entirely to the simply cuboidal cells of the terminal end buds (b), and thus by 16.5 days *p.c.*, expression eems widespread due to the highly differentiated state of the lung (c). t = trachea; b = bronchiole; br = ronchi; e = terminal end bud.

TGFβ3 RNA expression is limited to the immature columnar epithelia cells of the proximal respiratory tract in the early mammalian lung [30, 31]. In contrast, TGFβ2 is seen in the cuboidal epithelium of the growing terminal end buds and in the differentiating alveolar epithelium [31].

Since TGFβ is a potent inducer of bronchial epithelial cell differentiation [66], it is tempting to speculate that this is the endogenous role of TGFβ2 in the differentiating alveoli. However, each of the three isoforms might also be required for control of epithelial growth or branching morphogenesis. At present, no protein localization data exist for TGFβ2 or β3, and no studies on embryonic pulmonary TGFβ function have been reported *in vivo* or in organ culture.

In the mammary gland, a different experimental approach has been taken. As described earlier, using plastic implants impregnated with active TGFβ *in vivo*, it has been found that this growth factor has profound effects on terminal end bud growth, which could not only affect extent of growth, but also the branching pattern of the organ [22]. The response of the epithelial cell was dependent on the exact cell type and position within the mammary gland. As yet, however, the endogenous isoform distribution of TGFβ RNAs or proteins has not been mapped for this developing organ.

Auto-Regulation of Epithelial Growth and Differentiation

Several circumstantial lines of evidence suggest that TGFβ2 might be endogenous auto-regulator or epithelial growth and/or differentiation. Glick *et al.* [74] have demonstrated that TGFβ2 levels are elevated *in vitro* and *in vivo* in keratinocytes treated with retinoic acid or calcium ions, which induce growth arrest and differentiation, respectively. Secondly, in the embryo, TGFβ2 RNA levels are very high in the differentiating epithelial component of several established structures. These include epithelia of the sense organs, lung alveolar epithelium [30, 31], hyperplastic nodules (rugae) of palatal oral epithelia [29], and supra-basal keratinocytes [28] (see Table 1). Furthermore, where it is possible to distinguish the differentiation and dividing cell compartments, it appears that the differentiated cells are the source of this growth factor. Bearing in mind the strong negative growth regulation exerted by TGFβs on epithelial cells, one might imagine a situation where TGFβ2, produced from differentiated epithelial cells, acts on the dividing cell compartment in a paracrine feedback loop, as suggested previously for TGFβ1 [68]. The localization of TGFβ2 protein within the epithelium *per se*, following retinoic acid treatment of adult mouse skin, would give support to this theory [74].

NEURONAL DEVELOPMENT

The presence of TGFβ2 RNA within the sensory epithelia of the ear, eye and nose (Table 1) could be correlated with differentiation of the epithelium, as discussed above, or be associated with the innervation of the epithelium. It is interesting that TGFβ2 RNA is expressed transiently in the ventral nervous system of the early mammalian embryo [30, 31], and that TGFβ2 and β3 proteins have been detected immunohistochemically in central and peripheral nervous systems [75]. Flanders *et al* [75] suggested that these growth factors are important in the regulation of neuronal cell migration and

differentiation and of glial cell proliferation and differentiation.

CONCLUSIONS AND PROSPECTS

In summary, it can be stated that, *in vitro*, each of the mammalian TGFβs possesses quantitative, if not qualitative, functional specificity, and, *in vivo*, that each isoform has a distinct temporal and spatial pattern of expression throughout mammalian embryogenesis. The extreme amino acid sequence conservation of individual isoforms between vertebrates, as compared with isoform variation with individual species, coupled with the conservations of embryonic expression patterns within mammals [30], would suggest that, indeed, each isoform has a distinct function *in vivo*.

To test this proposition, it is essential that more functional studies are carried out, for example using organ culture to examine epithelial–mesenchymal interactions, or transgenic mice to manipulate isoform expression or isoform function.

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